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SCHEDULE OF MEETINGS

INTERNATIONAL—NATIONAL—REGIONAL

1954

NATIONAL: UNITED STATES AND CANADIAN SECTIONS

September 7-10	Palmer House	Chicago, Illinois

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To Be Announced	Hotel Utah	Salt Lake City, Utah

1955

INTERNATIONAL

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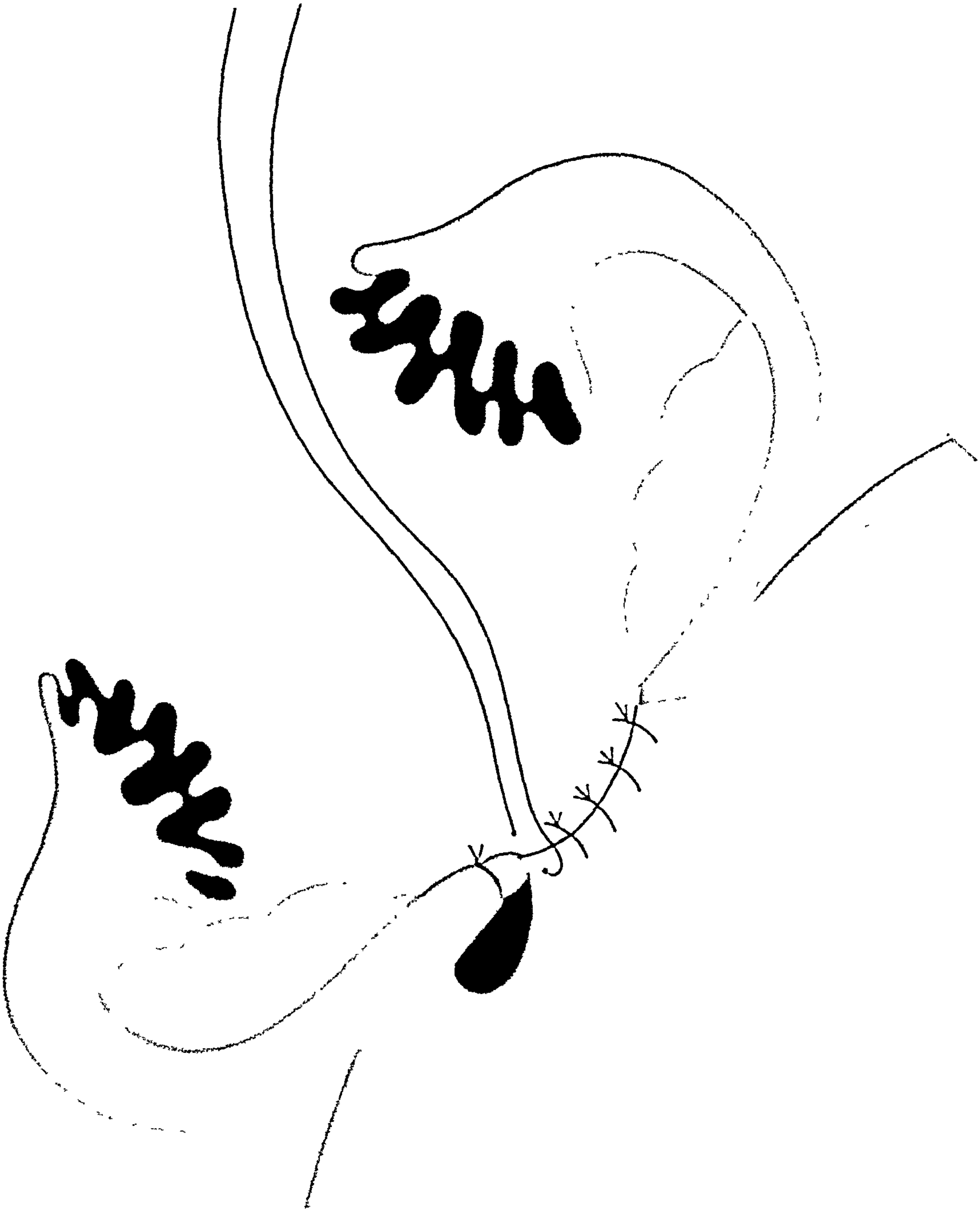

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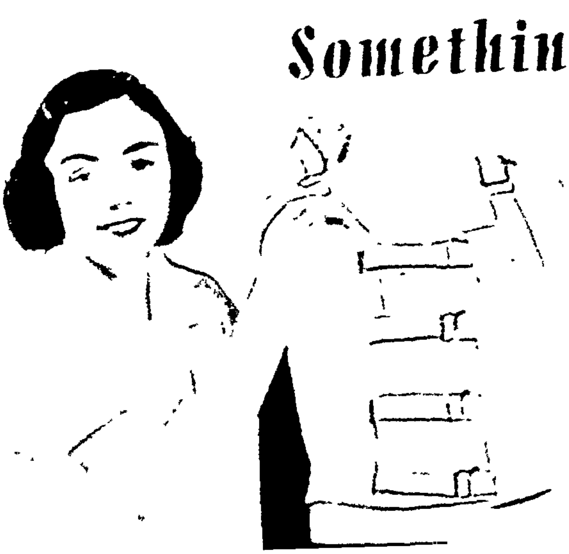

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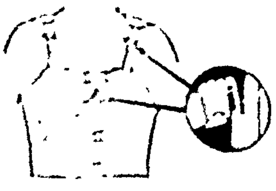

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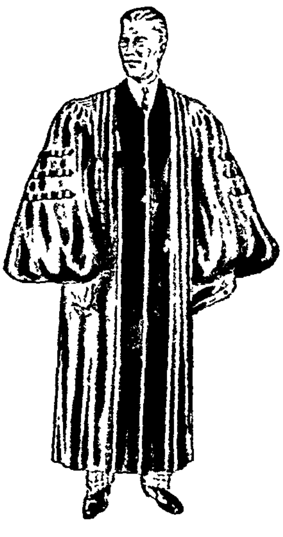

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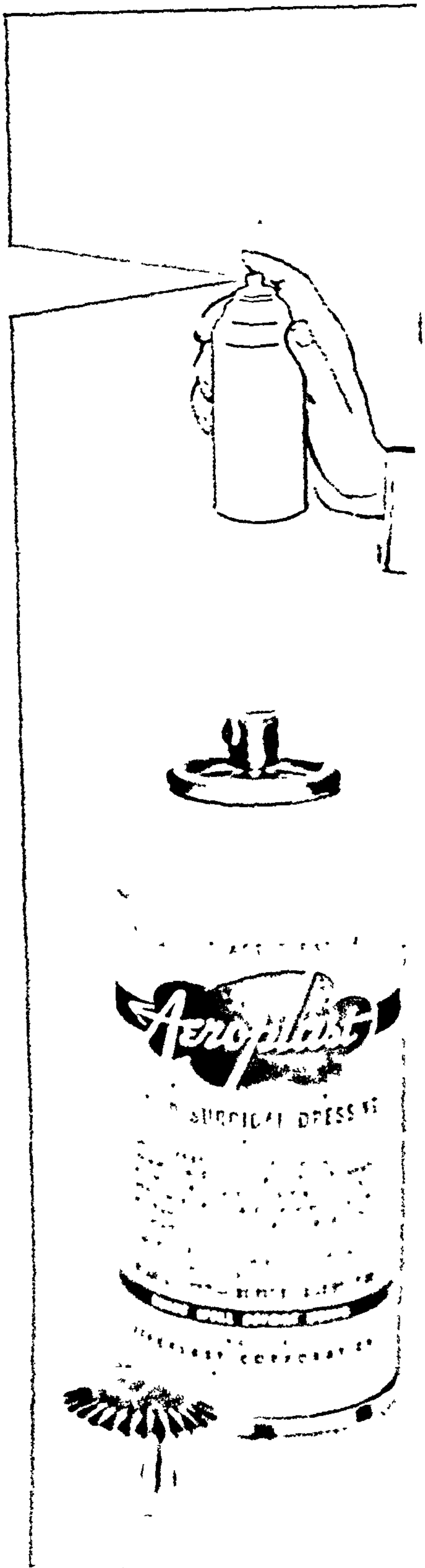

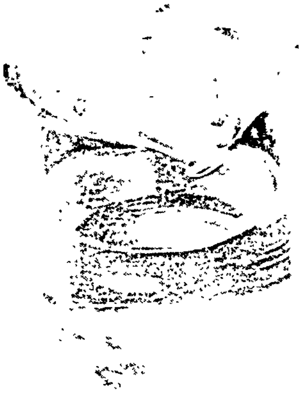

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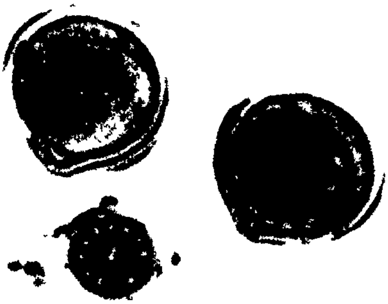

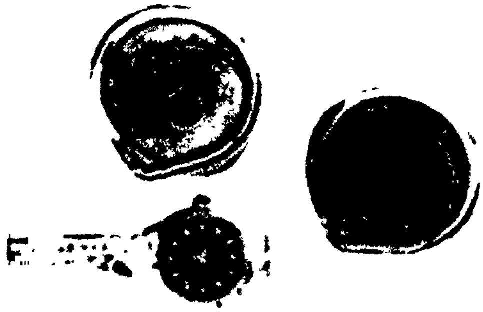

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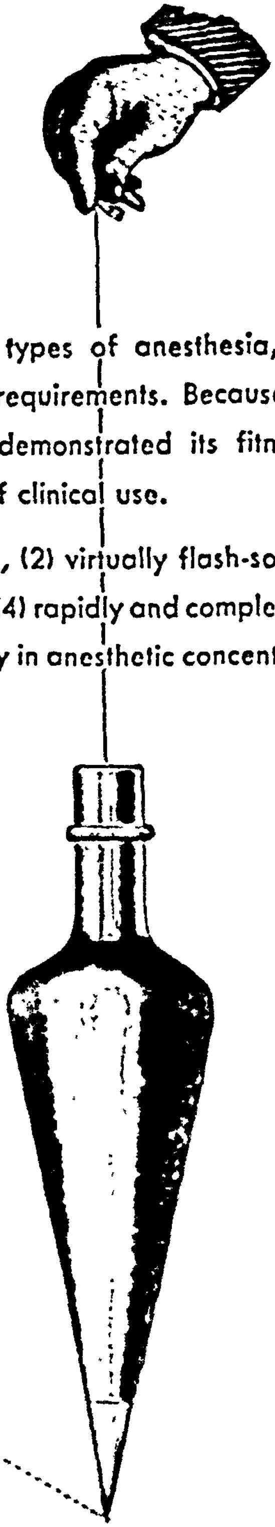

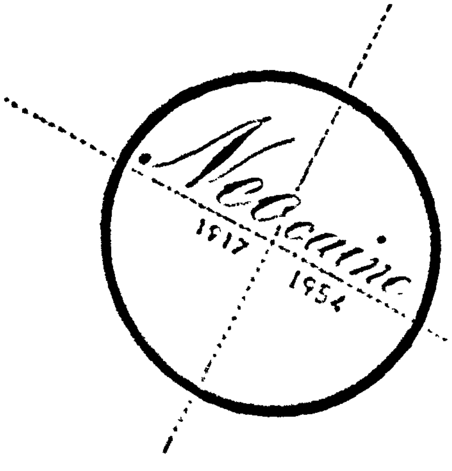

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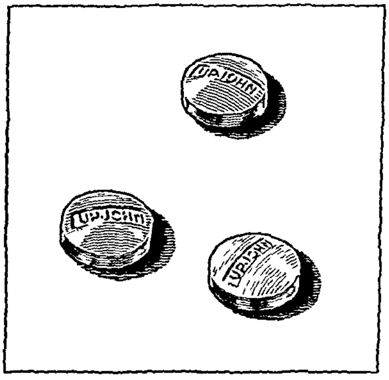

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COLLÈGE INTERNATIONAL DES CHIRURGIENS
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Original Articles

Chronic Thyroiditis: Review of Forty-Two Cases

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HALSTEAD, KANSAS

percentage of correct preoperative diagnoses than seems possible today. Patterson and Starkey,[1] in their review of 34 cases of chronic thyroiditis, made a correct diagnosis in 28 per cent of cases of the Hashimoto and Riedel types, but in neither case of giant cell thyroiditis. Malignant goiter was suspected in 55 per cent of cases of Riedel's goiter, in 9 per cent of cases of Hashimoto's disease and in 100 per cent of the DeQuervain group.

In 187 operative cases of chronic thyroiditis at the Lahey Clinic,[2] incorrect diagnoses were made in 87 per cent of the cases of Hashimoto's disease and 88 per cent of the cases of nonspecific thyroiditis. The clinical diagnoses in our 42 cases are listed in Table 1. In chronic thyroiditis [illegible] suspected in only 10 per cent [illegible] malignant goiter. Over one [illegible] were thought to have [illegible] toxic nodular goiter, and

TABLE 1.—*General Data*

Patient							Pathologic Picture		
Case	*Age*	*Sex*	*Symptoms*	*Preoperative Diagnosis*	*Preoperative Treatment*	*Operation*	*Gross*	*Microscopic*	*Follow-up*
1	35	F	Goiter 15 years; last year right side tender; bilateral, firm, nodular goiter	Nodular toxic goiter		6-9-42 Bilateral thyroidectomy	2 lobes, 86 Gm.; capsule glistening; many nodules, 2 to 11 mm. diam.; internodular tissue hard, grayish-white, very thick	Nodules composed of large colloid filled follicles; in thyroid tissue proper lymph follicles and diffuse lymphocytic infiltration; many small acini with macrophages; few Hürthle cells	1-14 to 4-2-49 in hosp. (schizophrenia); 5-18-50 cholesterol 385 mg. after discontinuing thyroid extract; adenomas in pyramidal process
2	30	F	Goiter 7 months; very nervous last 3 months; shortness of breath; lost 8 lbs. during last 2 months; slightly obese	Nodular non-toxic goiter		1-19-44 Bilateral resection	2 lobes and wide isthmus, 21 Gm.; glistening capsule; poorly circumscribed node, 7 mm., in right lower pole; thyroid tissue grayish; many brown islands 5-6 mm. diam.	Follicles of medium and large size; diffuse lymphocytic infiltration and lymph follicles; many small acini with single and syncytial monocytes	3-16-44, neck sore, very nervous, tires easily. Pulse 86. Takes thyroid extract. Follow-up letter returned 3-20-52, no address
3	33	M	Goiter 2 years; lost 11 lbs.; very nervous; headache; firm, bandlike goiter; pulse 92	Hyperplastic goiter		4-29-44 Bilateral resection; adhesion to surrounding	2 lobes, large isthmus; 15 Gm.; cut surface brown; follicles visible; many gray dots and streaks	Large follicles with well stained colloid; phagocytes in many lumens, also replacing epithelial wall; few lymphocytes and no lymph follicles	Paralysis left vocal cord. Thyroid extract 11-28-44, nervous, headache, pulse 84. March 1952, gained 50 lbs. Nervous. Thyroid medication
4	17	F	Goiter 5 years; last year marked increase in size; constipated; no heart symptoms; regular menses; bilateral goiter, firm, uneven; pulse 62	Nodular goiter	Iodine 4 years	6-20-44 Bilateral resection; no adhesions	62 Gm.; glistening cut surface; no nodules; cut surface dry, grayish amber, firm	Most acini small, cuboid epithelium, mostly empty except with syncytial cells; macrophages in larger colloid-filled lumens; many lymph follicles and diffuse lymphocytes	Paralysis left vocal cord. 8-1-44, no menstruation since operation. Tires easily. Follow-up letter returned March 1952, no address

	1-2-41 Bilateral resection	2 lobes and isthmus, 16 Gm.; 9 cm., glistening capsule; no nodules, cut surface light amber; thick [illegible] cysts	Most follicles of medium size; high cuboid epithelium, thin colloid; in one area large fibrosis with narrow tubules and strands of thyroid cells; few lymphocytes, few colloidophages	7-17-41, soreness and stiffness of neck. Paralysis of left laryngeal nerve. March 1952, pain left side of neck and swelling left side of thyroid. No medication
Propylthiouracil 6 weeks before 4 weeks 15 years [illegible]	6-28-50 Extensive scar tissue; bilateral resection	30 Gm.; capsule torn owing to adhesions; diffuse, moist, light-brown tissue; many grayish dots measuring 1 to 1.5 mm.	Many large follicles with cuboid epithelium and light red colloid; many small acini, some without colloid but with groups of mononuclear cells; lymph follicles with germinal centers	8-30-50, puffiness of eyelids, feels cold. Thyroid extract. May 1951, has gained 18 lbs.; dry skin. PBI 3.2 mg.
	8-4-51 Bilateral resection	2 lobes connected with isthmus, 22 Gm.; in latter, nodule 4 x 5 mm.; in right lower pole, node 11 x 6 mm.; in left lobe, node 3 x 1.8 cm.; grayish-red thyroid tissue	Follicles of small and medium size, with well stained colloid; many groups of lymphocytes, few lymph follicles; in some follicles mononuclear phagocytes; few oxyphilic cells	Oct. 1951, gained 14 lbs. since operation. Tired all the time. Pain in neck. Cholesterol 339 mg. thyroid extract. Felt better one month later
Iodine several months	8-11-51 Bilateral resection	2 lobes and isthmus, 30 Gm.; capsule glistening; 12 mm. node in right lower pole; thyroid tissue granular, brownish	Large follicles with well stained colloid; small colloid adenoma; many lymph follicles; groups of mononuclear cells in many lumens; one lobule composed of oxyphilic cells	10-11-51, gained 16 lbs. Tires easily. Hardness in neck. Thyroid extract. 12-22-51, feels better; doing own housework
	9-5-51 Bilateral resection	27 Gm.; grayish-amber tissue	Medium sized follicles; colloidophages; follicles and groups of lymphocytes	

TABLE 1.—*General Data (Cont'd.)*

Case	Patient		Symptoms	Preoperative Diagnosis	Preoperative Treatment	Operation	Pathologic Picture		Follow-up
	Age	Sex					Gross	Microscopic	
10	43	F	Goiter 6 months; regular menses; nodule on left side	Nodular goiter (adenoma?)		12-5-51 Bilateral resection	12 Gm.; thin fibrous capsule; light amber tissue, no nodes; many whitish dots 1-3 mm.	Large follicles, many lymph follicles; mononuclear cells in several lumens; fibrosis in one area with loss of thyroid tissue	1-24-52, well. No weight gain. Pulse 84
11	30	F	Choking spells if lying flat, nervousness; 16 lb. loss of weight last 5 mo.; regular menses; bilateral nodular goiter, very hard; BMR —23; pulse 72	Hashimoto's hypothyroidism		5-2-42 Bilateral resection of hard white tissue	5 Gm.; two pieces of hard grayish-amber tissue	No thyroid tissue; dense fibrous tissue, many lymph follicles and diffuse infiltration with lymphocytes and plasma cells	Bilateral paralysis of laryngeal nerve. 4-1-43, swelling of face, pain in back of head. Skin dry. Thyroid extract. 1-23-47, sleepy spells. Pulse 64. 10-16-49, cont. thyroid extract
12	40	F	Feb. 1942, hard left lobe removed, 26 Gm., clin. diag. malignant goiter; histologic diag. Riedel's struma; Sept. 1942 large rt. lobe; dyspnea; hoarse	Recurrent Reidel goiter		9-7-42 Hard tissue removed from trachea	6 Gm.; coarse granular surface; hard, grayish-brown, solid	Mostly fibrous and muscle tissue; small thyroid lobule; small acini with colloid. Low cuboid epithelium. Monocytes and syncytium in several lumens	April 1950, respiration difficulty. Stenosis of trachea (x-ray). Vocal cords, normal motility. Thyroid extract
13	66	F	March 1944, right lobectomy. (Exophthalmic goiter, 28 Gm.) May 10, 1944, sec. spec., nodular, large left lobe. Tremor, exophthalmus; pulse 70	Exophthalmic goiter	Lugol's 2 weeks	5-16-44 Left lobectomy; some adhesions	38 Gm. Adhesions on capsule. Multinodular cut surface with thick whitish septum. Nodules 2 to 11 mm.	Large follicles; few colloidophages and syncytial cell groups; in septums, dense fibrous tissue with some lymph follicles and lymphocytes; small fetal adenoma	Normal motility of vocal cords. 8-16-44, good condition. Letter from daughter March, 1952; patient died July, 1950, of "cancer"
14	53	F	Goiter and nervousness 2 years; loss of weight; rapid heart; firm nodular goiter	Nodular goiter	Lugol's 1 year	10-24-44	16 Gm.; 2 lobes and isthmus. Adhesions on capsule. Whitish amber hard tissue; small cyst, (4 mm.) in right lobe	Many small acini with high epithelium, many phagocytes and syncytial cell groups; many lymphocytes and several lymph follicles; dense fibrous tissue; two giant cells	12-31-44, swelling of face and hands. Thyroid extract. 2-4-48, still takes thyroid extract. Good condition. March 1952, continuing thyroid medication

[illegible]	[illegible]	[illegible]	[illegible]	[illegible]		7-24-45 [illegible]	[illegible] Gm. [illegible] right [illegible] left lobes; whitish hard diffuse [illegible]; small brown areas 1-3 mm.	Extensive fibrosis; narrow strands of thyroid cells; groups of large follicles, with colloid; one group of oxyphilic follicles. Monocytes and syncytial cells in several lumens; diffuse infiltration with lymphocytes; few lymph follicles	7-26-45, puffiness of eyelids. 11-1-45, gained 10 lbs. Proloid. 11-30-45, swelling of face disappeared. Letter March, 1952: patient died of cerebral hemorrhage in January 1948
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	Lugol's 6 weeks	11-10-45 White, hard; bilateral tissue removal	7 Gm. Thick adhesions, right and left lobe. Grayish-white, few small brownish areas, 1-6 mm. in diameter	Marked fibrosis; extensive lymphocytic infiltration. Few lymph follicles. Several large colloid-containing follicles. Monocytes and syncytium in several lumens; small group of oxyphilic cells	Discharged Nov. 18, 1945
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	Lugol's many years	5-12-44 Bilateral almost total resection	98 Gm. Right and left lobe and large nodular isthmus, with 4 cm. node. On cross section 2 cystic areas in isthmus, 8 and 11 mm. respectively. Both lobes had dry, grayish-red cut surface	Diffuse and nodular proliferation of oxyphilic cells; some monocytes and syncytia in lumens; fibrosis in some areas; some lymphocytic infiltration; few lymph follicles	5-18-44, right vocal cord stationary. Thyroid extract, 11-16-45, still needs thyroid extract. 1-7-52, "feeling fine" and still takes thyroid medication
[illegible]	[illegible]	[illegible]	[illegible]	Recurrent hyperplastic goiter		5-19-50 6 Gm. tissue removed from over larynx	6 Gm. Circumscribed nodes of amber tissue, 5-6 mm.	Large follicles, some with oxyphilic cells, some with papillae; many lymphocytes, some lymph follicles; strands of large oxyphilic cells; many intrafollicular phagocytes; no fibrosis	6-5-1950, feeling well. Some swelling of thyroid region
[illegible]	[illegible]	[illegible]	[illegible] Bilateral goiter	Early hyperplastic goiter; probable adenoma		7-18-50 Bilateral resection	32 Gm. 2 lobes and large isthmus. Lobulated surface. No nodes on cut surface. Moist, glistening light amber cut surface	Many oxyphilic cells forming small acini and solid cords; in many lumens, phagocytes; many lymph follicles	Paralysis left vocal cord. August 1950, swelling of face, constipation. Thyroid extract. April 1951, satisfactory; still takes thyroid extract

TABLE 1.—*General Data (Cont'd.)*

	Patient						Pathologic Picture		
Case	Age	Sex	Symptoms	Preoperative Diagnosis	Preoperative Treatment	Operation	Gross	Microscopic	Follow-up
20	35	F	Goiter 1 week, noticed by physician, 8 months by patient. Nervous, fast heart, weight gain. Large, firm, bilateral goiter. Pulse rate 84. Regular menses	Nodular colloid goiter		7-15-50 Bilateral resection	80 Gm. Two lobes and wide isthmus. Moist, grayish-amber tissue, without nodes. Firm. Node in pyramidal process, 8 x 12 mm.	Colloid nodule in pyramidal process. In both lateral lobes and small acini, many with phagocytes and syncytial cells. Many cells large and oxyphylic. Many lymph follicles and much diffuse lymphocytic infiltration	9-8-50, patient gained some weight. Puffiness of eyes. Thyroid extract. 5-17-51, BMR —12. Some anemia. Thyroid extract continued
21	31	F	Goiter 1 year. nervous 3 years. Difficulty in swallowing 1½ years. Bilateral smooth goiter	Diffuse nontoxic goiter		2-3-50 Adhesions to surrounding bilateral resection	24 Gm. 2 lobes and isthmus. Thin glistening capsule. No nodes on cross section. Yellowish-white tissue, very firm. Distinct interlobular septums	Many lymph follicles and diffuse lymphocytic infiltration. Most acini small, with oxyphilic epithelium; many containing phagocytes, and syncytium	3-3-50, gaining weight. Frontal headache. June 1950, gained 10 lbs. February, 1951, condition satisfactory
22	52	F	Pressure sensation in neck, 2½ years. Gained weight (15). Bilateral firm nodular goiter	Nodular colloid goiter, possibly malignant		1-26-50 Bilateral resection	102 Gm. Two lobes. Deep grooves from trachea on right lobe, which was partly retrotracheal. Hard grayish white tissue. Distinct fibrous septums	Very small acini; high cuboid oxyphilic epithelium; phagocytes and syncytial cells in narrow colloid-free lumens; many lymphocytes and lymph follicles	4-5-50, heart pounding, face puffy. Pulse 66. Thyroid extract. April 1951, no signs of myxedema
23	39	F	Goiter 8 weeks, sudden onset tenderness. Swelling of hands and feet. 20 years earlier had toxic symptoms. Weight loss 20 lbs. Regular menses. Firm goiter	Nodular nontoxic goiter		5-8-50 Bilateral thyroidectomy	43 Gm. 2 lobes and broad isthmus. Diffuse, grayish-red, moist cut surface. Small node (8 mm.) and cyst 9 mm. in isthmus	Small acini, many oxyphilic large cells. Most lumens, no colloid but phagocytes and syncytial cells. Infiltration with lymphocytes, marked, many lymph follicles; solid strands oxyphilic cells	7-22-50, swelling of face. 10-30 to 11-3-50, hospital, neurosis, BMR +2, cholesterin 150 mg.; 5-19-51, feels well
24	58	F	Goiter 1 year. Heart beat fast for 2 months. Menopause 1938. Pulse rate 84. Fine tremor	Nodular colloid goiter; mildly toxic		8-12-50 Bilateral resection	97 Gm. 2 lobes and isthmus. Diffuse grayish-red cut surface of both lobes, 7 mm. nodule in isthmus	Acini of various size; mostly small; many oxyphilic cells; solid strands of oxyphilic cells; abundant lymphocytic infiltration; many lymph follicles	10-6-50, thyroid extract. No symptoms of hypothyroidism

				[illegible] Bilateral [illegible]	[illegible] Cut surface [illegible]	Many small acini and solid cords of oxyphilic cells; phagocytes and syncytium-like cells in many lumens; few large colloid-filled follicles; very many lymphocytes and lymph follicles	12-12-50, gaining weight. Pain, back of neck. Pulse 54. Dry skin, facial puffiness. Cholesterol 140 mg. Thyroid extract. 4-21-51, constipation, tightness of neck
[illegible]	[illegible]	[illegible]		1-18-51 Bilateral resection	24 Gm. 2 lobes and large isthmus. Lobular surface. Cut surface brownish-red; several dry gray areas, 2 to 3 mm.; firm	Large and small follicles; cuboid epithelium; few follicles with papillary columnar epithelium; many oxyphilic cell strands and small acini; few phagocytes and syncytia; much lymphocytic infiltration and many lymph follicles	June 1951, choking sensation. Constipation. Thyroid extract. January 1952, nervous, sleepless; thyroid extract continued
[illegible]		Nodular [illegible] goiter		4-24-51 Bilateral resection	24 Gm. 2 lobes and isthmus. Lobular, firm whitish tissue	Small acini; oxyphilic large cells. Very many phagocytes, syncytia, and lymph follicles	8-23-51, adhesions right
[illegible]	[illegible]	Adenomatous goiter		8-23-51 Adhesion right lobe; bilateral resection	215 Gm. 2 lobes and isthmus. Deep tracheal groove on right posterior surface; grayish-red, very firm tissue	Thin fibrous septums; small acini, colloid filled. Oxyphilic large cells; in many acini, monocytes and syncytia. Some lymph follicles; small oxyphilic cell adenoma	Postoperative auricular fibrillation. Left vocal cord stationary. 1-21-52, nervous; stomach trouble, feels weak
[illegible] F	[illegible] BMR [illegible] Regular [illegible] Tremor. Bilateral [illegible]	Toxic [illegible] goiter	Propylthiour. Pot. iodide	8-29-51 Bilateral thyroidectomy, subtotal	12 Gm. 2 lobes and isthmus. No nodes. Light reddish-amber moist tissue; very firm	Small acini with monocytes in colloid-free lumens. Most cells oxyphilic, large; many lymphocytes and lymph follicles; several large follicles	11-21-51, pain in back of neck. Weight increase of 20 lbs. Heart rate 96

TABLE I.—*General Data (Cont'd.)*

	Patient						*Pathologic Picture*		
Case	*Age*	*Sex*	*Symptoms*	*Preoperative Diagnosis*	*Preoperative Treatment*	*Operation*	*Gross*	*Microscopic*	*Follow-up*
30	61	F	Choking sensation, rapid heart, nervousness. Enlargement of neck. Difficulty in swallowing. Dry skin. Pulse 84. BP 210/100, Nodules right and left lobe	Fetal adenoma		9-26-51 Adhesions to muscle and fascia, softening of trachea	32 Gm. 2 lobes and isthmus. No nodes. Grayish-red, very firm tissue	Very many lymphocytes and lymph follicles; small acini with dense colloid; large oxyphilic cells forming many small acini and strands. Some macrophages and syncytia	Oct. 31, 1951, gained weight. Feels cold. Sleeps well
31	22	F		Exophthalmic goiter		11-3-51 Subtotal thyroidectomy	40 Gm. 2 lobes and isthmus. Yellowish-gray surface and brownish-gray cut surface; very firm	Many lymph follicles; most of thyroid acini small; groups of monocytes in many lumens; many oxyphilic cells	
32	53	F	Goiter 2 years. Menopause 1½ years earlier. Right lobe diffusely en-enlarged. Firm node in left lobe	Adenoma, left; colloid enlargement, right		2-13-51 Adhesions; bilateral resection	38 Gm. 2 lobes and isthmus. In left lobe, tumor 2.5 cm.; in left upper pole, tumor 6-8 mm. Indistinct nodules 3-6 mm. In right lower pole grayish-amber node 15 mm. in diameter	Many large follicles; small acini and strands composed of large oxyphilic cells; some lymphocytes and lymph follicles. Diagnosis: Nodular and diffuse colloid goiter, Hashimoto	Sept. 9, 1951, pain left side of neck. Thyroid extract since 4-11-51
33	54	F	Goiter 2-3 years. Choking sensation. Tonsillectomy, 1943. Nervous. Weight gain, 30 lbs. Menopause 1950. PBI 4.4. Neck tender	Hashimoto's disease; early hypothyroidism		12-13-51 Bilateral resection	33 Gm. 2 lobes and isthmus. Lobules separated by septums; distinct, light grayish-amber tissue	Very many lymphocytes and lymph follicles; most acini small; mostly large eosinophilic cells; single and syncytial cells in many lumens	Jan. 6, 1952, patient feels well
34	53	F	Nervous, exhausted, 2 years. Fullness of throat, diagnosed two weeks earlier as goiter. High BP for 9 years. Arthritis 1935. Menopause at 39. Nodular, bilateral firm goiter	Nodular toxic goiter	Lugol's; roentgen therapy	9-20-40 Bilateral resection	22 Gm. Surface of right and left lobe covered with fibrous adhesions and fat tissue; firm; cut surface grayish-brown, dense; scattered foci, 2-4 mm., of brown tissue	(a) Areas with colloid-filled follicles. Low cuboid epithelium; slitlike acini with monocytes and syncytia; high epithelium (b) Areas of fibrosis with giant-cell tubercles; dense fibrous stroma and diffuse infiltration with lymphocytes and plasma cells; no colloid-containing follicles	10-2-40, right vocal cord stationary. Thyroid extract. 7-9-41, swelling of face. In hospital at times with coronary disease. 12-23-44, difficulty in speaking. Thyroid extract. Died July 1951, invalid 6 years

					[illegible] Bilateral [illegible]	(a) Right lobe 5 x 4 x 3 cm., left lobe 4 x 2 x 1.5 cm. Solitary adenoma in right lobe, 2.5 cm.; amber, partly cystic. Other thyroid tissue coarsely granular, firm and tender	(a) Adenoma, hyaline center with trabeculae and small acini. Large colloid-filled follicles in periphery. Thyroid tissue proper medium-sized follicles; many lymph follicles. Monocytes and syncytia	
			[illegible]		4-21-41 Hard tissue in front of trachea removed; bilateral resection	(b) 5 Gm. Left and right lobe. Grayish, firm	(b) Few areas of colloid-filled acini, some with monocytes and syncytia; extensive lymphocytic infiltration and some lymph follicles; one small lobule of oxyphilic cells; tubercles with giant cells	(b) April 25, 1941, both vocal cords stationary. Tracheotomy July 1941. Dyspnea, cough. January 1942, shortness of breath, swelling of eyelids. Right vocal cord stationary. Thyroid extract
[illegible]	[illegible]	[illegible]	[illegible] 17 years [illegible] Bilateral, [illegible]	Hyperplastic goiter	9-29-43 Bilateral resection	16 Gm. 2 lobes and isthmus. Marked adhesions on surface. Colloid node, 7 mm., in left lower pole. Other tissue grayish-white, smooth; no visible follicles	Large follicles in adenoma; in thyroid tissue proper, occasionally small follicles with colloid; follicles with giant cell wall, several layers of histiocytes; scanty lymphocytic infiltration; extensive fibrosis	Right vocal cord limited motility. Thyroid extract. Pressure to neck. Letter, March 1951, takes thyroid extract, working. Some enlargement left side of neck, choking sensation. Much better since operation
7	47	F	[illegible] in right side of neck. [illegible] Pain on swallowing. Shortness of breath. Lost 2 lbs. T. 100.2 F., pulse 120. Bilateral hard goiter, left lobe very tender	Subacute thyroiditis	10-26-43 Adhesions of muscles; bilateral resection	21 Gm. 2 lobes and isthmus. Thick fibrous capsule; hard, grayish-amber tissue. 1-2 mm. follicles visible	Increased fibrous stroma; many lymphocytes and plasma cells. Large and medium-sized follicles with cuboid epithelium; in many lumens, monocytes; many acini without epithelium, lined with histiocytes; few tubercles with giant cells, mostly only epitheloid tubercles	Left vocal cord stationary. Thyroid extract. March 1949, gained weight. Pressure and pain in neck region. No enlargement of thyroid

in one-third of our cases the preoperative diagnosis was toxic goiter. We have no experience in the aspiration biopsy with the Silverman needle as recommended by Crile; however, since the inflammatory changes were often observed in focal areas, it is our opinion that this method is much less reliable than a histologic study of operative specimens.

Age and Sex.—The average age of patients with mild chronic thyroiditis was 36.5 years, that of patients with Hashimoto's disease 50.6 years and that of patients with Riedel's struma 44.3 years. In the DeQuervain group the average age was 49.8 years. The average age of the pa-

TABLE 2.—*Clinical Diagnosis*

	Thyroiditis	*Diff nontoxic*	*Nod nontoxic*	*Diff toxic*	*Nod toxic*	*Fetal adenoma*	*Cancer*
A. Mild Thyroiditis 10 Cases		3	2	1	3	1	
I. Riedel, 6 cases	1		2	2			1
II. Hashimoto, 17 cases	1	1	4	3	1	4	3
III. DeQuervain, 9 cases	2			1	1	5	
Total, 42 cases	4	4	8	7	5	10	4

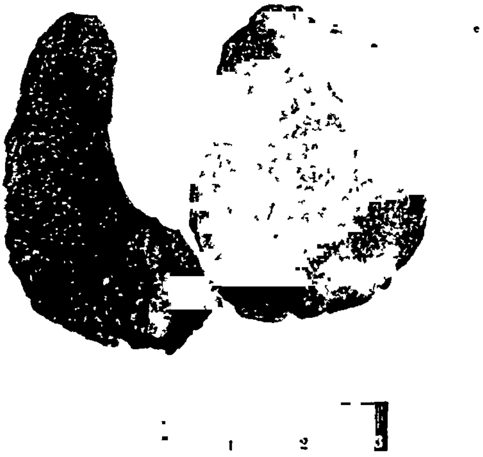


Fig. 1.—Mild thyroiditis in 50-year-old woman. Weight of specimen, 27 Gm. Grayish dry areas in moist amber cut surface indicate foci of colloidophagy and lymphocytic infiltration, the essential characteristics of thyroiditis.

TABLE 3.—*Age and Sex*

	Youngest	*Oldest*	*Average*	*Sex*
A. Simple thyroiditis	17	50	36.5	1 M 9 F
I. Riedel	29	66	44.3	6 F
II. Hashimoto	21	66	50.6	17 F
III. DeQuervain	37	61	49.8	9 F
Total	17	66	45.9	1 M 43 F

TABLE 4.—*Clinical Symptoms*

	A Simple	*I Riedel*	*II Hashimoto*	*III DeQuervain*
Enlargement of neck	10	6	17	9
Nodular goiter	7	3	10	6
Firm or hard	5	3	5	4
Dyspnea	1	2	1	4
Choking		1	4	1
Dysphagia			2	2
Pressure		1	3	2
Pain, tenderness	1		6	4
Headache	1			2
Hoarseness	1	1	1	
Fatigue	2		1	3
Nervousness	5	3	6	4
Weight gain		1	3	
Weight loss	4	2	3	5
Rapid heart	2	2	6	4
Tremor		1	2	1
Myxedema			4	
Perithyroiditis during operation	1	6	4	9
Nodules in surg. spec.	2	1	1	2

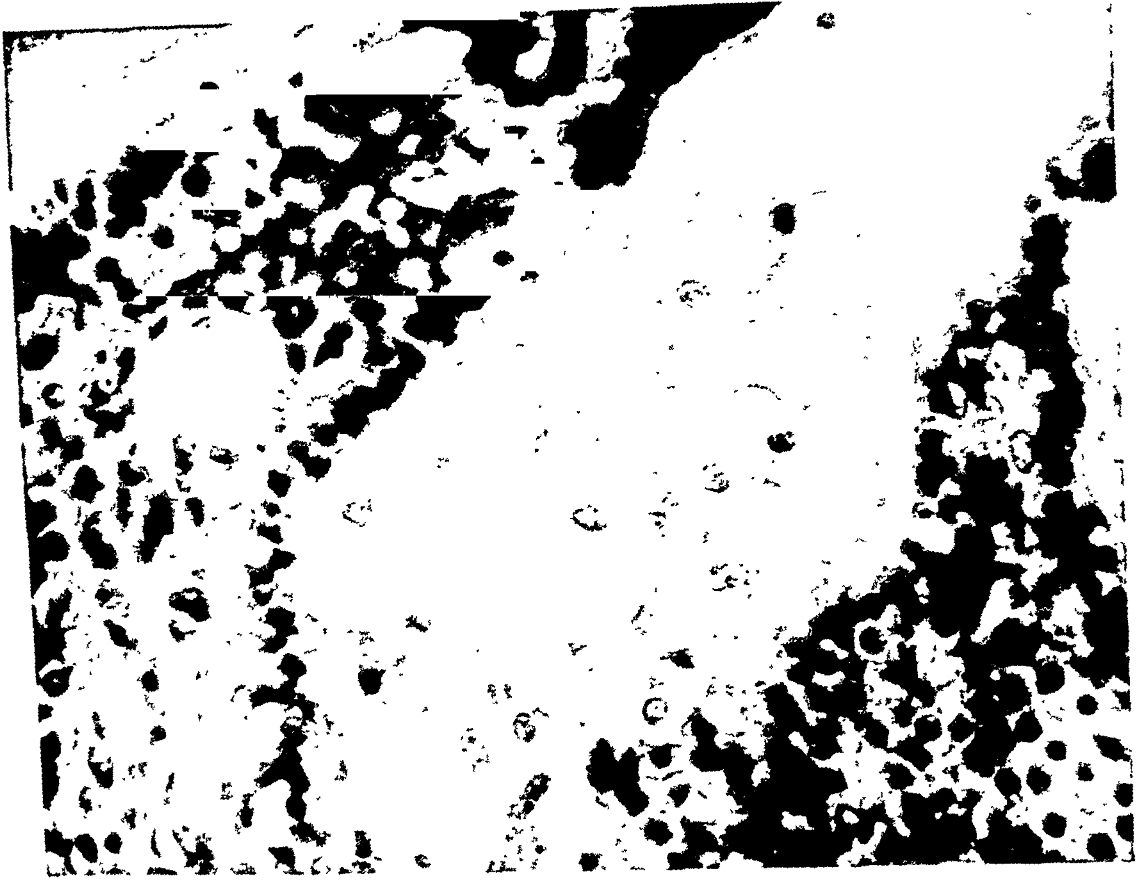


Fig. 2. Mild thyroiditis. The cells in the colloid are monocytes, which are attracted by the lipid in the colloid. × 580.

tients with the three common types of thyroiditis, therefore, were essentially similar and were not significant enough to help in the differential diagnosis. All of our patients were white women except 1, who had mild thyroiditis.

Clinical Signs and Symptoms.—Clinically, enlargement of the neck was present in all of our cases of thyroiditis. To the examining physician the enlargement appeared nodular in 26 instances, firm in 10 and extremely hard in 7. Pain or tenderness in the thyroid gland occurred in 1 of the 10 cases of mild thyroiditis, in none of the cases of Riedel's struma, in 6 of 17 cases of Hashimoto's disease, and in 4 of the [illegible] patients with DeQuervain's type of thyroiditis. Pressure symptoms were noted in [illegible] 7 [illegible] 12 [illegible] Hoarseness was present in 1 patient with early thyroiditis, and also occurred once in association with Riedel's and Hashimoto's goiters. Dyspnea was noticed 8 times, dysphagia 4 times and a choking sensation 6 times.

Duration of Symptoms.—The duration of symptoms in our first group averaged eight and four-tenths months; in the Riedel group eight and eight-tenths months, in the Hashimoto group one year, and in the DeQuervain group one and seven-tenths months.

Treatment.—We have not treated any of our patients with irradiation, although there are some reports of good results in the literature. We did not use roentgen therapy: first, because we agree with Marshall that additional fibrosis produced by irradiation may well increase constriction of the trachea and also destroy re-

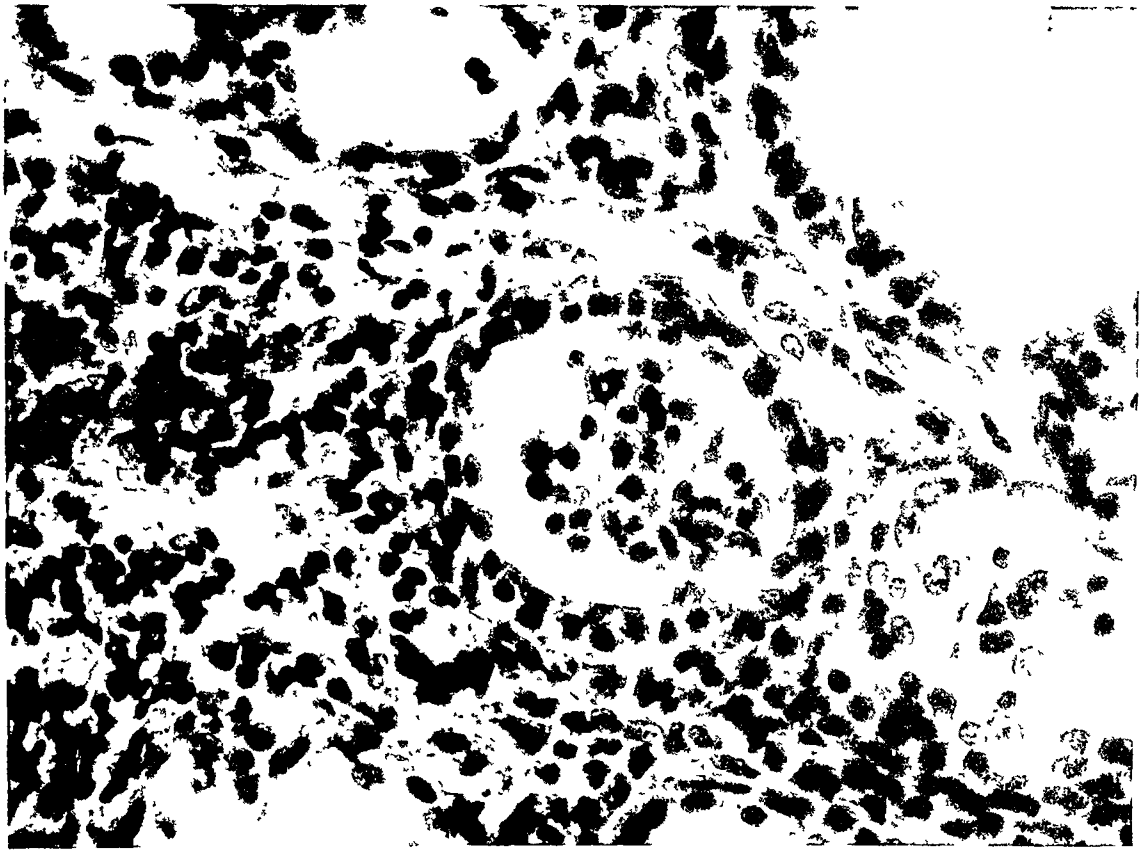


Fig. 3.—Photomicrograph of follicle with monocytes and lymphocytes. The mitosis in one of the intrafollicular cells proves that they are not necrotic, desquamated epithelial cells × 580.

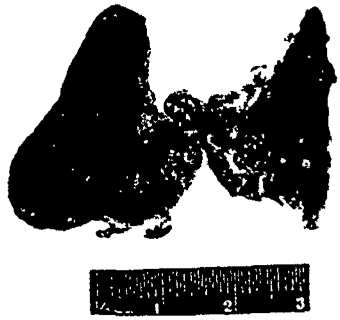
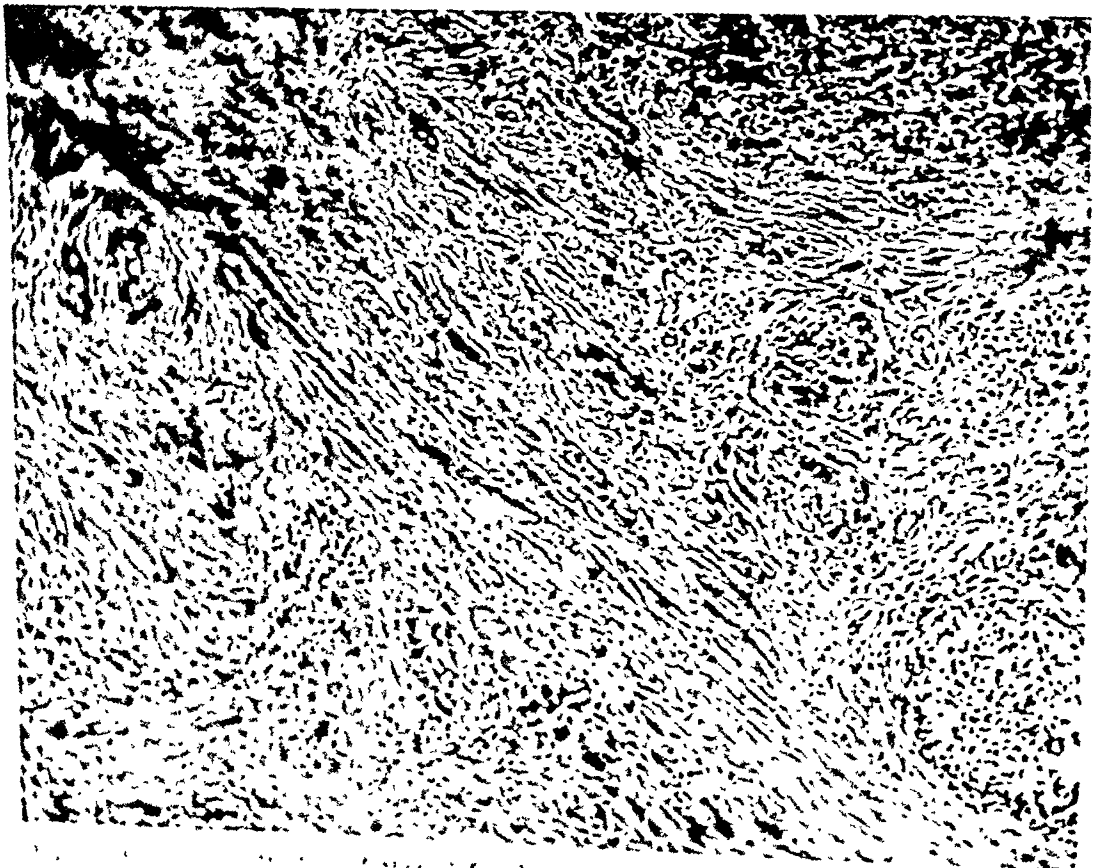


Fig. 4 (Case 16).—Riedel's disease in a woman aged 47. Specimen weighed 27 Gm. Dense superficial adhesions, whitish cut surface.

maining thyroid epithelium, and second, because we wished to be sure of our diagnosis. To us the principal reason for surgical intervention is relief of pressure symptoms and tracheal constriction. In the presence of Riedel's or DeQuervain's type of the disease radical resection not only may be difficult because of the stony hardness of the gland, but may result in serious injury to the parathyroid glands and to the recurrent laryngeal nerves. Release of the viselike pressure on the trachea by removal of the isthmus and limited resection of the lateral lobes is all that we attempt. On the other hand, a subtotal bilateral lobectomy is the method we prefer for Hashimoto's disease.

In Table 5 the weights of the resected thyroids are tabulated. The average weight was 13.8 Gm. for Riedel's struma, 19.2 Gm. for DeQuervain's type and 59.9 Gm. for Hashimoto's disease. In the presence of chronic inflammation of longer duration the thyroid tissue tends to decrease in volume, except in cases of Hashimoto's disease. In studying the last-mentioned condition we found by actual cell count that the greater part of the gland is the result of proliferation of large oxyphilic cells, and decidedly less the result of lymphoid hyperplasia. Only in cases of this type, therefore, is one dealing with a true goiter, i.e., a cellular proliferation. Since inflammatory involvement of surrounding structures is rare in patients with Hashimoto's disease, the danger of damaging laryngeal nerves and parathyroids during operation is not greater than in the presence of a noninflammatory goiter. The fear of postoperative hypothyroidism is of no concern to patients with Hashimoto's disease, as oxyphilic cells are functionally inert and hypothyroidism will develop with or without operation.

Cause of Thyroiditis.—Table 7 reveals the differences in microscopic tissue structure associated with the four types of chronic thyroiditis. In our first group, intrafollicular macrophages and lymphocytic aggregations in the stroma are the main histologic features, while Riedel's goiter shows, in addition, a marked increase of fibrous tissue with destruction of glandular elements, and Hashimoto's disease is characterized by proliferation of



[illegible] Riedel's struma. [illegible]

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THE DISTRIBUTION OF LIPID AND PHOSPHOLIPID IN PAPER ELECTROPHORESIS OF THE SERUM LIPOPROTEINS IN NORMAL SUBJECTS AND IN PATIENTS WITH ATHEROSCLEROSIS

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IN SUPPORT of the view that atherosclerosis may be a metabolic disease numerous investigators have found an association with increased beta lipoproteins of serum,[1-4] with increases in the cholesterol content of the beta lipoproteins,[5, 6] with increases in the low density lipoproteins, and with an increased *serum cholesterol:phospholipid ratio.[8-10] Most of the evidence indicates* atherosclerosis to be associated with abnormal increases in the beta lipoproteins. Changes in the alpha lipoproteins are slight and consist of slight decreases in the lipid and cholesterol.[3, 5]

While cholesterol, as a more insoluble component of the lipids, has been considered to play a dominant role in the metabolic disturbance of lipids promoting atherogenesis,[11] its status as a single causal entity has frequently been questioned, perhaps mainly because in any series of patients with coronary disease there are many *with normal concentrations of cholesterol in the serum. Increasing* attention has therefore been given to the other lipids[12] of serum and to the lipid protein relationships[3] in terms of possible lipid stabilizing effects.

Recently, for the same reason, the phospholipids have also come under scrutiny.[13-16] The constancy of the cholesterol:phospholipid ratio has been emphasized,[17] and it has been shown that no less than a 1:1 molar ratio of phospholipid to free cholesterol exists in the plasma at all times.[16] In experimental canine atherosclerosis, Davidson and associates[13] have found phospholipid to increase at the average rate of but one mole for every five moles of cholesterol. A consequence of such findings has been the growth of a concept that the available phospholipid determines the amount of cholesterol which can be kept in solution in the plasma and that relative increases of cholesterol may result in instability of the lipid solution.[18]

It, therefore, seemed desirable to investigate changes in the phospholipid content of the serum beta lipoproteins in normal subjects and in those with atherosclerosis with the hope that such comparison might give a better understanding of the nature of lipid disturbance in this disease. This study has been based on a simplified method[19] utilizing paper electrophoresis as a means of separation *of the beta and alpha lipoproteins in multiple strips of paper, some of which*

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were stained directly for estimation of lipid distribution between the two groups, and the remainder stained for phospholipid after differential extraction and removal of the other lipid components. This technique has permitted a gross estimation of the beta and alpha lipid fractions, of the beta and alpha phospholipid in these fractions, and indirectly, the amount of phospholipid in the total lipid of serum.

MATERIALS AND METHODS

All chemical determinations were done on blood serum obtained in the fasting state, 14 to 16 hours after a light evening meal. Each serum was refrigerated at 5° C. and the various chemical and electrophoretic determinations accomplished within 24 to 48 hours. Several groups of persons were studied. These consisted of 20 young men and 22 young women, all presumably normal and healthy; 18 older persons, also normal by the usual examinations and routine tests; 20 subjects, of ages comparable to the older age group, who had had recent episodes of myocardial infarction and were 1 to 3 weeks in the recovery phase at the time of testing; 10 with uncomplicated diabetes mellitus; 3 with nephrosis; 5 with essential hypercholesterolemia; and 2 with myxedema.

Serum cholesterol and esters were determined by the method of Schoenheimer and Sperry.[20] The method of Fiske and Subbarow[21] was used for the determination of lipid phosphorus and the factor of 25 for its conversion to phospholipid. Triglycerides were calculated from the difference between the total fatty acids[22] and the sum of the estimated phospholipid and cholesterol ester fatty acids. Total serum lipids were calculated as the sum of the values for free cholesterol, estimated cholesterol esters, phospholipid, and triglycerides.

A previously described modification[19] of the technique of Kunkel and Slater[6] for paper electrophoresis of serum lipoproteins which permits the simultaneous use of multiple strips of Whatman No. 1 filter paper, was employed throughout this study. A 0.02 ml. sample of the serum being tested was applied to each of the strips in a similar manner and location. The entire procedure of electrophoresis, drying of strips, and subsequent staining of lipids was standardized and accomplished in the same way for each determination. Ten or more of the dried strips from each serum electrophoretic run were treated with boiling acetone saturated with magnesium chloride to extract all lipids other than the insoluble phospholipid. These strips, along with at least 6 other unextracted strips from the same serum run, were subjected to the same staining procedure with 1 per cent Sudan Black B in 50 per cent ethanol (triple filtered) in one large tank. At the end of the 12-hour staining period, the strips were washed by successive immersion in 4 containers of 40 per cent ethanol for 15 minutes each. Since there was some fading of these dyed strips on exposure to bright light, all strips were dried and kept in the dark until manually scanned with a photoelectric densitometer using a 590 mμ filter. From the curves relating optical density and distance along the strip the areas corresponding to the beta and alpha albumin lipid zones were measured by planimetry and the percentage distribution of the lipids in the 2 lipoprotein zones calculated. With careful technique and attention to detail, the reproducibility of this method is within ± 5 per cent for total lipids and ± 9 per cent for phospholipid.

RESULTS

In view of the fact that the findings here reported indicate both the total lipid and phospholipid distribution in the beta and alpha lipoproteins and therefore apply only to the lipid moiety of these fractions, the presentation of results by application of usual terminology becomes complicated and cumbersome. In the following discussion it has been found advantageous to refer to the total lipid in the beta and alpha lipoproteins as the beta and alpha lipids, and to the phospholipid in each fraction as the beta and alpha phospholipid.

Distribution of Total Lipid and Phospholipid.—

A. *Young men and women:* The young women of this group were from 19 to 30 years old and the young men were 16 to 32 years old. There was no family history of diabetes or hypercholesterolemia in any of the subjects The data on these two groups are listed in Table I. The average values for the group of men for serum total lipid and for total and free cholesterol were slightly greater and the values for phospholipid slightly lower than the corresponding values for women. In these young people, phospholipid represented 41 per cent of the serum lipids in young women and 34 per cent of those in young men

TABLE I. DISTRIBUTION OF SERUM LIPID AND PHOSPHOLIPID IN YOUNG MEN AND WOMEN BETWEEN 16 AND 32 YEARS OLD

DETERMINATION	NORMAL WOMEN (22 SUBJECTS) AVERAGE	NORMAL WOMEN (22 SUBJECTS) RANGE	NORMAL MEN (20 SUBJECTS) AVERAGE	NORMAL MEN (20 SUBJECTS) RANGE
Chemical Findings (mg. per cent)				
Total cholesterol	152	90 228	158	102 191
Free cholesterol	37	12 79	43	25 56
Cholesterol esters	115	75 165	115	72 156
Lipid phosphorus	8 6	7 0 11.4	8 3	6 5 10 1
Phospholipid (factor 25)	215	170 285	208	163 253
C-P ratio	0 71		0 76	
Triglycerides	82	0 169	162	96 217
Total lipid	527	424 656	614	467 694
Phospholipid per cent of total lipid	41	33 46	34	27 39
Electrophoretic Findings (per cent)				
Total lipid distribution				
Beta zone	71	58 83	76	65 85
Alpha zone	29	17 41	24	15 39
Phospholipid distribution				
Beta zone	67	59 83	64	53 84
per cent of total lipid	31	18 43	26	15 40
per cent of beta lipid	44	26 64	34	26 47
Alpha zone	33	17 54	36	16 47
per cent of total lipid	15	5 25	14	8 20
per cent of alpha lipid	52	31 80	60	44 87
Phospholipid per cent of total lipid	43	31 53	40	32 49
Calculated Distributions				
Beta lipid (mg. %)	374	259 525	466	314 565
Beta phospholipid (mg. %)	144	98 188	133	87 212
Phospholipid per cent of beta lipid	39	28 53	29	20 38
Alpha lipid (mg %)	154	85 258	148	92 239
Alpha phospholipid (mg %)	71	40 154	75	41 110
Phospholipid per cent of alpha lipid	46	33 64	51	35 69
Beta alpha lipid ratio	2.4	1 1 4 9	3 3	1 6 5 6
Beta alpha phospholipid ratio	2 0	0 9 4 9	1 8	0 9 5 2

The sera of young women averaged more alpha lipids (154 mg per cent) and less alpha phospholipid (71 mg per cent) than was found in the sera of young men (148 and 75 mg per cent, respectively). The sera of young men averaged 24 per cent greater beta lipids (466 mg. per cent, compared with 374 mg per cent), and nearly 10 per cent less beta phospholipid (133 mg. per cent, compared with 144 mg per cent) than was found in the sera of young women These differences in the lipid and phospholipid distribution in the two main lipoprotein fractions of serum from young people of different sexes, are shown in Fig. 1 where the values for the beta lipids are related to the serum total lipid of each individual examined, and in Fig. 2, where the values for the beta phospholipid are related to the serum phospholipid in each instance.

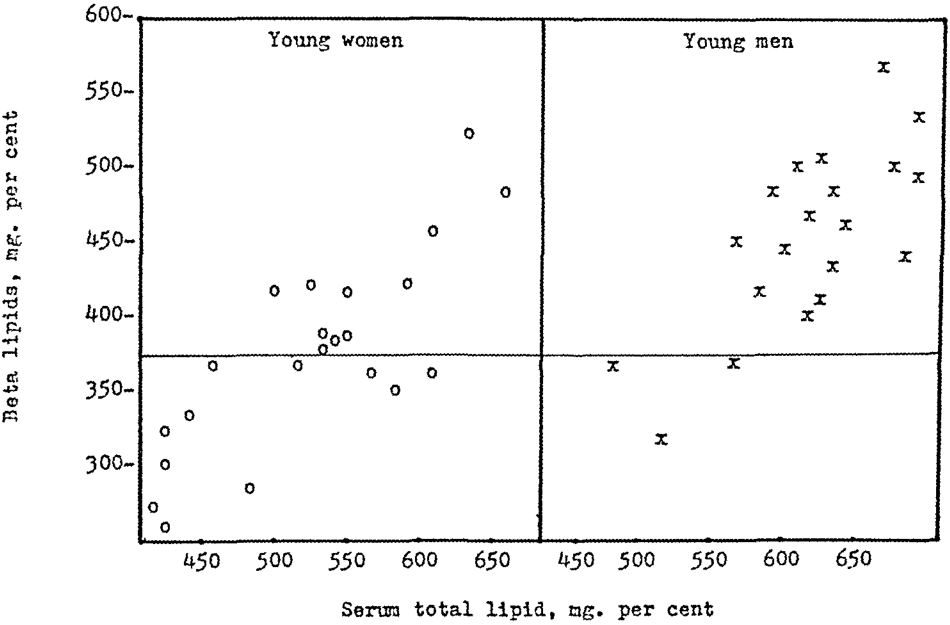

Fig. 1.—Comparison of the beta lipid values for sera of young women and men. The majority of young men have beta lipids in excess of the average for young women, represented by the horizontal line.

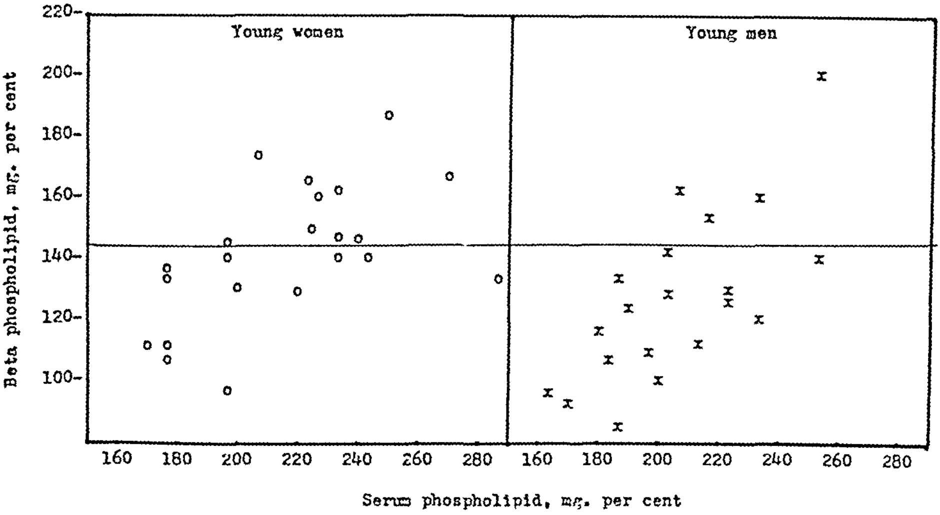

Fig. 2.—Comparison of the beta phospholipid values for sera of young women and
The majority of young men have beta phospholipid values less than the average for
women, represented by the horizontal line.

These findings indicate the sera of young men to have greater beta lipids with less beta phospholipid, and less alpha lipids with more alpha phospholipid than is found in the sera of young women, with the greatest difference being evident in the beta lipoproteins

B *"Normal" older group* In this group of 18 subjects, there were 10 postmenopausal women and 8 men, ranging in age from 40 to 66 years The persons comprising this group had been hospitalized for routine examinations or had been admitted for functional complaints None showed evidence of organic disease or overt atherosclerosis The average findings for this group are listed in the second vertical column of Table II for comparison with the findings for young women, selected as the reference group

Chemical analyses of the sera of this group showed an increase in total lipids and slight increases in cholesterol and phospholipid in comparison with the sera of young women After electrophoretic separation, 82 per cent of the serum lipids migrated with the beta lipoproteins, compared to the 71 per cent distribution for the group of young women, and 73 per cent of the phospholipid accompanied the beta lipids From calculated concentrations this group showed greater amounts of beta lipids and phospholipid than was found in the group of young women, the phospholipid percentage composition of the beta lipids was 32 per cent and comparatively lower Both the alpha lipids and phospholipid were less in this group than in the group of young women

Clinically normal older persons appear, therefore, to have increased beta lipids and phospholipid, with the former being disproportionately increased and the latter amounting to a difference of 7 per cent less than its composition of the beta lipids of young women In reciprocal fashion, there were less alpha lipids and phospholipid than was found in the sera of young women No sex difference in the distribution of these lipids was detected in this group of older people

C *Persons with known coronary atherosclerosis* This group of 20 subjects, all men and 38 to 63 years old, represented instances of recovery from recent myocardial infarction Three of the group were recovering from a second attack Six were having intermittent angina pectoris All patients with diabetes mellitus and those with suspected essential hypercholesterolemia were excluded from this group As noted in Table II, the chemical analyses of the sera, when averaged, showed moderate increases in all of the lipid components as compared with those of young men and particularly of young women Phospholipid content averaged 35 per cent of the total lipid, approximating the percentage composition of the sera of older persons and of young men, but notably less than the 41 per cent composition found in young women

Electrophoretic separation of the serum lipids of this group disclosed a greater proportion (83 per cent) of the total lipid in the beta fraction than was found in the groups of normal subjects Both the alpha lipids and alpha phospholipid values were decreased The average beta lipids of 640 mg per cent represents a 70 per cent increase over that for young women Thus, while the beta phospholipid was somewhat increased, this increase was disproportionate,

TABLE II. DISTRIBUTION OF LIPID AND PHOSPHOLIPID IN YOUNG WOMEN, IN OLDER PERSONS, AND IN CONDITIONS ASSOCIATED WITH ATHEROSCLEROSIS

DETERMINATION	NORMAL YOUNG WOMEN (22 SUBJECTS)	NORMAL OLDER PERSONS (18 SUBJECTS)	RECENT MYOCARDIAL INFARCTS (20 SUBJECTS)	UNCOMPLICATED DIABETES MELLITUS (10 SUBJECTS)	NEPHROSIS (3 SUBJECTS)	ESSENTIAL HYPERCHOLESTEROLEMIA (5 SUBJECTS)	UNCOMPLICATED MYXEDEMA (2 SUBJECTS)
Chemical Findings (mg. per cent)							
Total cholesterol	152	155	225	213	336	438	378
Free cholesterol	37	47	63	64	97	141	119
Cholesterol esters	115	108	162	149	239	297	259
Lipid phosphorus	8.6	9.8	10.7	11.2	18.0	14.6	18.4
Phospholipid (factor 25)	215	245	268	280	450	367	460
C-P ratio	0.71	0.70	0.85	0.77	0.75	1.09	0.82
Triglycerides	82	208	187	172	706	203	748
Total lipid	527	681	770	752	1642	1210	1760
Phospholipid per cent of total lipid	41	36	35	27	27	30	26
Electrophoretic Findings (per cent)							
Total lipid distribution							
Beta zone	71	82	83	85	94	89	87
Alpha zone	29	18	17	15	6	11	13
Phospholipid distribution							
Beta zone	67	73	74	76	88	75	71
per cent of total lipid	31	31	30	35	21	23	18
per cent of beta lipid	44	38	36	35	22	26	21
Alpha zone	33	27	26	24	12	25	29
per cent of total lipid	15	12	10	10	3	6	7
per cent of alpha lipid	52	55	50	51	63	46	55
Phospholipid per cent of total lipid	43	43	39	39	24	28	25
Calculated Distributions							
Beta lipid (mg. %)	374	560	640	650	1560	1080	1530
Beta phospholipid (mg. %)	144	179	198	213	398	275	326
Phospholipid per cent of beta lipid	39	32	31	33	26	25	21
Alpha lipid (mg. %)	154	121	130	102	82	130	230
Alpha phospholipid (mg. %)	71	66	70	67	52	91	134
Phospholipid per cent of alpha lipid	46	55	54	66	68	70	58
Beta alpha lipid ratio	2.4	4.6	4.9	6.4	19.0	8.3	6.7
Beta alpha phospholipid ratio	2.0	2.7	3.5	3.1	7.7	3.0	2.4

All figures represent average determinations.

and its percentage composition of the beta lipids averaged 31 per cent. This was considerably less than was found in the group of young men and is conspicuous in view of the gross increases in the other beta lipid components. Triglycerides, which were also increased in the sera of this group, represented some portion of the latter, since it migrates in large part with the beta lipids.[73] The alpha lipids and phospholipid of this group were both decreased in actual average amounts below those of young women. Phospholipid appeared to make up somewhat more than half of the alpha lipids in these subjects.

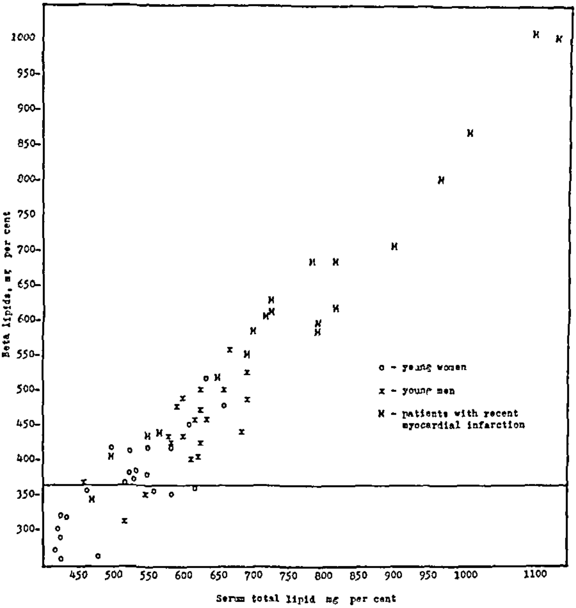

Fig. 3.—The relationship of beta lipids to serum total lipid in young women, young men and in patients with coronary atherosclerosis. The horizontal line represents the average beta lipid concentration for young women. Values for the majority of young men and for all but one of the patients with recent myocardial infarction fall above this line.

These data, therefore, indicate the sera of patients with coronary atherosclerosis to have considerably increased beta and slightly decreased alpha lipids giving a beta alpha lipid ratio of 4.9, *as compared to the ratio of 2.0 found in* young women. The phospholipid distribution, however, followed the same pattern found in the sera of young men and of the older group of normal persons, i.e., while the beta phospholipid increased moderately as compared with the sera of young women, this increase was not in proportion to the great increase in the beta lipids as a whole. Thus, the beta phospholipid of this group was 20 per cent less than it would be if it were increased in the same ratio as in young women.

Figs. 3 and 4 are scatter diagrams showing the relationship of beta lipids to serum lipids and of beta phospholipid to serum phospholipid for the individual members of this group as well as those of young men and women. The deranged content of the beta lipids with regard to phospholipid is thus emphasized.

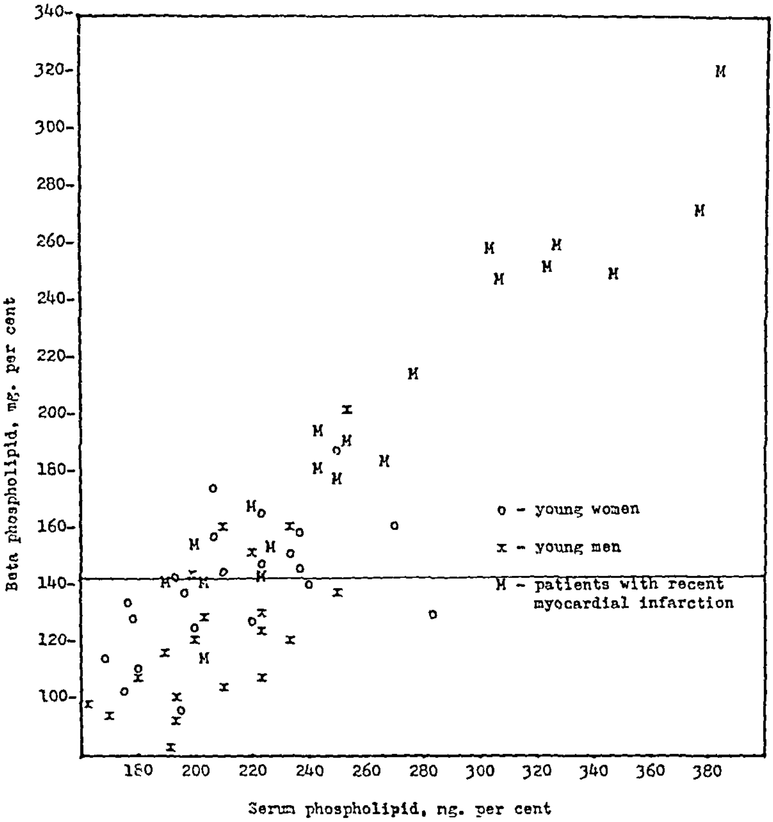

Fig. 4.—The relationship of serum and beta phospholipid in young men and women and in patients with coronary atherosclerosis. The horizontal line represents the average beta phospholipid concentration for the sera of young women. The values for most of the sera of young men fall below this line.

D. *Diseases with high incidence of atherosclerosis*: 1. *Diabetes mellitus*: Eight instances of this disease, uncomplicated by ketosis, infection, or overt coronary disease, comprised this group. By chemical analysis, all of the serum lipid components were increased over the normal, particularly the serum phospholipid which averaged higher than that of the preceding group with coronary atherosclerosis. As shown in the listing of results from this group inTable II, there were increased amounts of total lipid and phospholipid in the beta lipids of the sera examined, yet phospholipid represented but 33 per cent of this fraction

The alpha lipids were decreased but phospholipid was present in nearly normal amount as compared with that for young women. Some decrease in the other alpha lipid components is thus indicated to be present in diabetes. The beta-alpha lipid ratio was nearly threefold that of young women.

2. *Nephrosis.* The 3 subjects selected for this group had typical nephrotic syndromes with varying degrees of edema, hypoalbuminemia, proteinuria, and hypercholesterolemia. The nephrosis in all subjects was believed to be secondary to chronic glomerulonephritis. The abnormal beta lipoproteins of this group of sera migrated with an increased mobility, often encroaching on the alpha lipid fraction and making exact separation and subsequent analysis frequently difficult by the method employed. The greatly increased lipid content of these sera was reflected in an increase in all components, including triglycerides. From the data listed in Table II, the greatest portion of this increased lipid is seen to be present in the beta lipoproteins which contained 94 per cent of the total lipid and 88 per cent of the phospholipid which was present in the serum. By actual calculation, however, phospholipid comprised but 26 per cent of the greatly increased beta lipids, or considerably less than that of any of the other groups studied. Reciprocally, the alpha lipids and phospholipids were decreased in actual amounts, with the latter appearing to make up 68 per cent of the alpha lipids of this group. One of these 3 subjects whose serum total lipid was 2 300 mg per cent, appeared to have an alpha lipid consisting almost entirely of phospholipid. This unusual result, verified on repeated testing, while perhaps representing some peculiarity of the method of extraction or of staining as it applies to those abnormal lipoproteins, indicates the alpha cholesterol and triglyceride to be greatly decreased in nephrosis. Further studies are in progress with an aim to clarify this finding, particularly since similar results have been observed in some instances of acute infectious hepatitis and obstructive jaundice, confirming the earlier observations of Kunkel and Slater.[6]

3. *Essential hypercholesterolemia.* The 5 subjects in this group, 3 men and 2 women, were from 32 to 54 years old. All had had angina pectoris or myocardial infarction. One had fairly extensive tendon deposits of xanthomatosis and 4 had xanthelasma. Another, a 32-year-old woman with severe angina pectoris which was present before and after an earlier episode of myocardial infarction, had a strong family history of probable hypercholesterolemia (3 siblings had died between the ages of 22 and 34 of acute heart disease), and has since died with a recurrent myocardial infarction. From chemical analyses of the sera, the markedly increased lipids reflected increases in cholesterol and its esters and, to a lesser extent, in the phospholipid. In comparison with the sera of nephrotic patients, there was much less phospholipid and triglycerides. While the beta lipids were some threefold increased over that for young women, the beta phospholipid was but twofold increased and comprised only 25 per cent of the beta lipids. Of equal interest was the finding that while the alpha lipids were slightly decreased below those of young women in terms of actual amounts present (130 mg per cent), the alpha phospholipid (91 mg per cent) was increased over that for young women and represented 70 per cent of the alpha

lipids. This increase in the alpha phospholipid was of about the same degree as previously noted in the sera of patients with diabetes and nephrosis. It is interesting that in this disease which is characterized by great increases in serum cholesterol, alpha cholesterol appears to be decreased.

4. *Hypothyroidism*: The 2 patients in this group were 48- and 56-year-old women. Their basal metabolic rates were minus 36 and 40 per cent, respectively. Each had the classical clinical picture of myxedema. The serum total lipid was increased to an extent nearly equal to the average found in nephrosis, the serum total cholesterol was greater than in the latter but less than that found in hypercholesterolemia, and there were increased triglycerides. One of these sera was lactescent. The serum phospholipid was increased in degree comparable to that of the nephrotic sera. In these 2 subjects, 87 per cent of the serum lipids were present in the beta lipoproteins, giving the latter a lipid content of 4 times that for young women. Only 71 per cent of the serum phospholipid migrated in this beta fraction, with the result that the beta lipid content of phospholipid was but 21 per cent. This was the lowest concentration for beta phospholipid found in any of the sera examined in this study. Of equal interest was the finding that the calculated alpha lipids in this disease is apparently increased some 50 per cent, and the alpha phospholipid nearly 90 per cent, over the values for normal young women. More than one-half of the alpha lipids appear to consist of phospholipid in myxedema. The beta-alpha lipid ratio was increased to about the same degree as that in diabetic sera. The sera of both patients, after 6 months' treatment with desiccated thyroid, showed more normal lipid distribution patterns of the type described for the older age group of persons.

Summary of Findings in Atherosclerosis.—Both these and other[5] results appear to indicate that the serum lipids in coronary atherosclerosis and in diseases with a strongly atherogenic tendency show distinct changes when they are examined in terms of their component distributions in the beta and alpha lipoproteins and when compared with a reference group of normal young women. The general pattern of these changes appears to be as follows:

1. The beta lipids, as a group, are increased greatly.
2. The beta cholesterol is increased moderately.[5]
3. The beta phospholipid is increased slightly.
4. The phospholipid percentage content of the beta lipids is decreased greatly.
5. The alpha lipids are decreased moderately (excepting myxedema).
6. The alpha cholesterol is decreased slightly.[5]
7. The alpha phospholipid is decreased slightly (excepting myxedema).
8. The phospholipid percentage content of the alpha lipids is increased slightly.
9. The beta-alpha lipid ratio is increased greatly.
10. The beta-alpha phospholipid ratio is increased moderately.

These changes are visualized in Fig. 5, which illustrates in terms of milligrams per cent concentration the actual amounts of total lipid and phospholipid found in the beta and alpha lipoproteins in each of the different groups studied The lower bars represent the alpha and the upper bars the beta lipids, the whole bar in each instance representing the total lipid of each fraction. The shaded portion of each bar represents the amounts of beta and alpha phospholipid, and the percentage composition of each fraction is numerically inserted. It is seen that even though there is an increase in the beta phospholipid, this increase is disproportionately less than the increase in the beta lipids, and that the latter, in atherosclerosis, contain relatively less phospholipid than is found in the sera of young women.

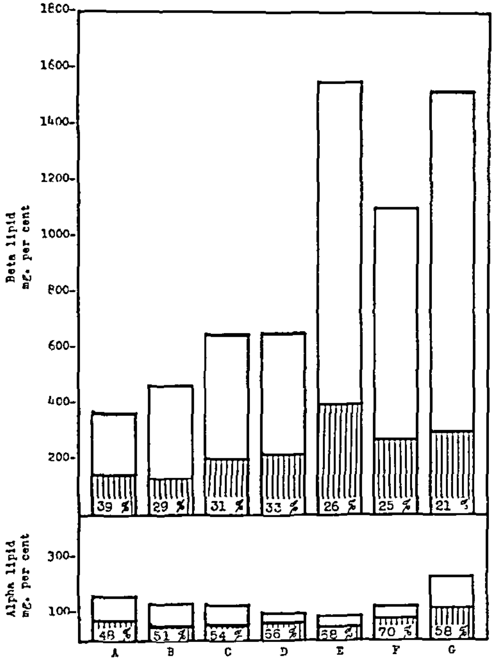

Fig 5—Relative lipid and phospholipid compositions of the beta and alpha lipoproteins in differing groups A Normal young women. B, Normal young men C, Patients with recent myocardial infarction D Patients with uncomplicated diabetes E, Nephrosis F Essential hypercholesterolemia, G, Myxedema. The portions of each bar representing phospholipid are striped and the percentage composition of each is numerically represented

DISCUSSION

Additional information concerning the pattern of abnormal lipid distribution in atherosclerotic sera has appeared as a result of this study. If one accepts the distribution of serum lipids and phospholipid of young women as the normal, or group of reference, then the sera of patients with coronary atherosclerosis have increased beta lipids which contain less than the normal ratio of phospholipid. A trend to this pattern was found in the sera of young men. Because of their potential importance, sex differences in the blood lipids have been sought by many investigators. Thus, Russ and associates[3] found young men to have more beta lipoprotein and less alpha lipoprotein than young women. Oliver and Boyd[5] found young men to have more beta cholesterol and less alpha cholesterol than young women. Nikkilä[1] and Fasoli,[2] by means of paper electrophoresis, had earlier demonstrated the same finding. Jones and co-workers[25] from a study of the low-density lipoproteins, found that 20 per cent of the men but only 2 per cent of the women between the ages of 31 and 40 have over 60 mg. per cent of the Sf 10-20 class of lipoproteins (believed to be of the beta fraction); a similar difference could not be demonstrated in older age groups. The data reported here indicate another sex difference involving the phospholipid composition of the beta lipoproteins, the latter, in young men, having more total lipid but relatively less phospholipid than those of young women.

This pattern is somewhat more distinct in the older age group of normal subjects of both sexes, the beta lipids being increased to a greater degree than the beta phospholipid. The trend is more distinct in the group of persons with coronary atherosclerosis and in other strongly atherogenic diseases. In these groups, the beta lipids are grossly increased whereas phospholipid constitutes a lesser percentage of this total. In all instances of patients with atherosclerosis, with the exception of myxedema, there are increases in the beta lipoproteins and decreases in the alpha lipoproteins of the serum. The significance of these reciprocal changes in the two lipoprotein fractions is unknown. Russ and coworkers[3] associated this finding with a diminished albumin content of the alpha fraction. The observations concerning the differences found in myxedema are unexplained.

The concept, revived by Boyd[26] and expressed by others,[8, 9, 13, 16] that phospholipids play an important role in maintaining stability of the plasma lipids and that lipid derangements resulting in decreased phospholipid concentration are associated with the development of atherosclerosis, provides a convenient interpretation of the findings of this study. It appears possible that this demonstration of a relative decrease in the beta phospholipid, occurring concomitantly with an increase in the other beta lipids—a disturbed distribution known to be present in atherosclerosis—signifies lessened stability in a system with a saturation limit.

It is known that the individual lipid fraction values, in the serum as well as in the two lipoprotein fractions, vary markedly from individual to individual and that there is a wide variation in values obtained from normal persons. The variations in these values in sera from patients with atherosclerosis is even more marked. Mann[24] has emphasized that the variability (of lipid values) within given sex and age group is very large, and that the measures of spread aro the mean are large, giving coefficients of variation of 30 to 50 per cent Therefore, in this, as in any study of blood lipids, average values should be interpreted as trends and not as absolute limits The statistical significance of the observed changes in the beta lipids and phospholipids in the groups of young men and women and in the group of patients with coronary atherosclerosis is indicated in Table III

TABLE III

GROUP	SEX	NUMBER OF SUBJECTS	BETA LIPID (MG PER CENT) Mean ± S D	p	BETA PHOSPHOLIPID (MG PER CENT) Mean ± S D	p
Normal young women	F	22	374 ± 54 3		144 ± 22 8	
Normal young men	M	20	464 ± 42 8	<0 01	133 ± 27 1	<0 5
Patients with atherosclerosis	M F	20	641 ± 137 9	<0 01	202 ± 62 0	<0 01

SUMMARY

1 A method, devised for the measurement of total lipids and phospholipids in the beta and alpha lipoproteins after electrophoretic separation of these serum fractions, has been applied to the sera of groups of normal young men and women, of older persons and of patients with coronary atherosclerosis Smaller groups of patients with diabetes mellitus nephrosis, essential hypercholesterolemia, and myxedema have also been included Cholesterol, phospholipid, triglycerides, and total lipids have been determined in the original serum and the total lipid and phospholipid in each of the two lipoprotein fractions

2 The sera of young men have more total lipid with relatively less phospholipid in the beta lipoproteins and less total lipid with relatively more phospholipid in the alpha lipoproteins than the sera of young women of comparable age

3 The sera of patients with coronary atherosclerosis and of patients with atherogenic diseases have greatly increased total lipid with relatively less phospholipid in the beta lipoproteins, and markedly decreased total lipid with relatively more phospholipid in the alpha lipoproteins when compared with the sera of young women

4 This pattern of abnormal lipid distribution in atherosclerosis has been shown to be most pronounced in the beta lipoproteins

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THE DIFFERING MECHANISMS OF ACTION OF MERCURIALS, CARBONIC ANHYDRASE INHIBITORS, AND CHLOROTHIAZIDE AS DIURETIC AGENTS

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THE precise mechanism(s) of action of diuretic agents remains an enigma. Histochemical and in vitro chemical data indicate that perhaps mercurials and sulfonamides inhibit specific enzyme systems for the activation of the ion exchange mechanism for sodium reabsorption. In an effort to supplement these data with reference to renal diuretic mechanisms, this study has been performed. It was speculated that the responses to 3 groups of diuretics—mercurials (meralluride), carbonic anhydrase inhibitors (acetazoleamide), and chlorothiazide—in the urinary excretion of sodium might reveal indirect evidence concerning their mechanism of action.

This study was inspired by preliminary work in dogs by Pitts,[1] which suggested that mechanisms of certain diuretics were "competitive" while others were "noncompetitive." The further exploration of this matter in human beings comprises the substance of this report.

MATERIALS AND METHODS

The subjects of the studies were men with well-controlled chronic congestive heart failure due to hypertensive cardiovascular disease. At the time of the study the patients presented no detectable edema. They were maintained on the metabolic ward and fed a diet containing 50 mEq. of sodium and 3,000 ml. of distilled water per 24 hours. Their average 24-hour excretion of sodium was 45 mEq. in the control state. On the morning of the study, and until its completion, the patients were not fed. They drank 1,000 ml. of distilled water from 6 A.M. to 7 A.M. and 250 ml. every hour until the study was completed. Urine, collected in 30-minute periods throughout the study, was analyzed for sodium, potassium (analyzed by means of a Beckman flame photometer), chloride,[2] and bicarbonate.[3] Beginning at 7 A.M. and throughout the study isotonic sodium chloride solution was infused intravenously at a rate of approximately 650 μEq of sodium per minute. The purpose of this infusion was to prevent depletion of body stores of sodium which might be reflected in decreased urinary sodium during the latter parts of the study, especially after potent diuretic administration. Three diuretics, meralluride (Mercuhydrin), acetazoleamide (Diamox), and chlorothiazide (Diuril), were *administered alone and in various combinations to determine* the effects on the excretion of the various electrolytes. Contemporary observations of glomerular filtration rate (inulin clearance) and renal plasma flow (para-aminohippurate clearance) were made. Each segment of this study was repeated 5 times and the results are *reported as the average values. In each value reported* the mean effect is at least 3 times the standard deviation.

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After four 30-minute control periods, a sustaining infusion of isotonic sodium chloride solution was administered at a rate of 650 μEq per minute and observations of the urinary electrolyte excretion were made for a total of 7 hours.

In order to determine the potency of the various diuretics in producing an increased sodium excretion, the next segment of the study was performed. The sustaining infusion of isotonic sodium chloride was started. After four 30-minute control periods the various drugs were given separately by intravenous injection, and the urinary electrolyte responses were observed in four 30-minute periods, or a total of 2 hours. These periods, selected after preliminary studies, revealed that maximum responses were seen in these periods. Although an occasional observation beyond this point revealed greater responses, this variation was not statistically significant. The effect of acetazoleamide on electrolyte excretion was observed at two dosages, 500 and 2,000 mg.; the dosages of meralluride were 20, 80, 160, and 320 mg. of mercury equivalent*; the dosages of chlorothiazide were 500, 2,000, and 4,000 mg.

In order to determine the possibility of competitive diuretic mechanisms other observations were made. The sustaining infusion of isotonic sodium chloride solution was started, and after 4 control periods meralluride was administered in a priming dose of 160 mg. mercury equivalent (4 ml.) and 160 mg. placed in the sustaining infusion. After a 2-hour period, during which time the excretion of sodium reached a maximum rate, a 500 mg. priming dose of acetazoleamide was given and the observations extended another 2 hours. Several days elapsed before the patient was used again as the subject of this study. However, since an ample quantity of sodium chloride had been provided during the study the body stores of sodium were only rarely depleted by the rather massive diuretic administration. Only rarely did a patient complain of muscular cramps, nausea, or diarrhea. In a subsequent study, the order was reversed. After a suitable control period, acetazoleamide was administered in a priming dose of 2,000 mg., and another 2,000 mg. was placed in the sustaining infusion of isotonic sodium chloride. After 2 hours, during which time the sodium response became maximal, meralluride (20 mg. mercury equivalent, 0.5 ml.) was administered intravenously as a priming dose and the observations continued as before. The purpose of these two studies was to observe the possible inhibition of sodium excretion by the second drug given after a maximum natriuretic effect had been obtained from the first drug.

Similar studies were done using meralluride 160 mg. (4 ml.) as a prime and 160 mg. as a sustaining infusion followed after 2 hours by 2,000 mg. chlorothiazide as a priming dose. Subsequently (on another day) chlorothiazide was given in a priming dose of 2,000 mg. (and 2,000 mg. sustaining) followed, after 2 hours, by a 80 mg. priming dose of meralluride (2 ml.).

Similar studies were performed using a priming dose of 2,000 mg. of acetazoleamide (and 2,000 mg. as sustainer) followed by 500 mg. chlorothiazide as a prime. On a subsequent day chlorothiazide was given first, 500 mg. prime and 500 mg. sustaining, followed by 2,000 mg. acetazoleamide as a prime.

A final study was performed using 160 mg. meralluride as prime (and 160 mg. sustaining), followed by 2,000 mg. acetazoleamide as prime (and 2,000 mg. sustaining), and both of these followed by a priming dose of 500 mg. chlorothiazide.

In each instance the dose of the first drug to be given was chosen as the one shown previously to produce a maximum sodium excretory response for that drug. The dose of the second drug was chosen as that one which was most nearly equal in potency (relative to sodium excretion) to the first drug. However, if the second drug evoked an increased sodium excretion, a dose one-half (or even one-fourth) the original dose was used to be certain that the factor of potency was not operative. On the other hand, if the second drug failed to evoke an increased sodium excretion, either the dose of the first drug was reduced by one-half (or one-fourth) or the dose of the second drug was doubled, whichever was more feasible.

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*Forty milligrams mercury equivalent equals 1 ml.

RESULTS

A The Response to Isotonic Saline Infusion (Fig 1)—Under the conditions of this study the intravenous infusion of isotonic sodium chloride solution produced a threefold increase in the urinary excretion of sodium (and chloride) which became apparent within 2 hours The rate of the infusion was approximately 650 μEq per minute of sodium and the excretory sodium rate was approximately 120 μEq per minute during the 7 hours of observation Bicarbonate and potassium excretion was not altered These patients, although previously edematous presented no evidence of edema at the time of the study and had been on a moderately restricted salt intake for some time During the remainder of the day and on the day following this procedure their sodium excretion was naturally greater than prior to the study as the kidney was able to excrete slowly the excess salt load presented by the infusion These data furnish the background upon which is superimposed the effect of various diuretics under identical conditions in subsequent studies

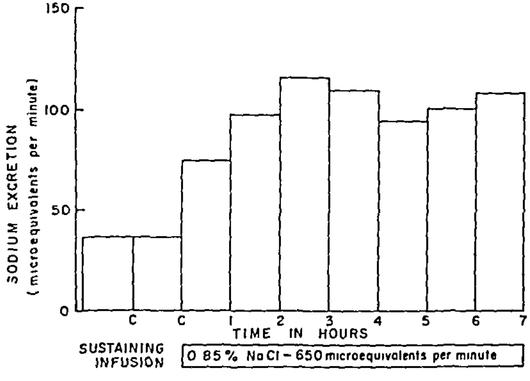

Fig 1—The response in sodium excretion to the infusion of isotonic saline in cardiac patients eating a diet containing 50 mEq sodium daily After two hours the sodium excretion is fairly stable during the continuous infusion of isotonic saline

B The Effect of Three Intravenously Administered Diuretics on the Excretion of Sodium and Other Electrolytes (Fig 2)—During the infusion of isotonic sodium chloride, the intravenous administration of 500 mg acetazoleamide produced a maximum sodium excretion of 300 ± 45 μEq per minute (control = 100 – 120) while a dose of 2 000 mg produced a rate of 340 ± 52 μEq per minute illustrating no significant difference between these two dosages The excretion rate of potassium was about 90 per cent that of sodium, bicarbonate excretion rate paralleled potassium, and the chloride excretion rate was depressed to less than the control value The intravenous administration of meralluride produced a sodium excretion of 301 ± 52 μEq per minute after 20 mg mercury equivalent (0 5 ml), 610 ± 72 after 80 mg , 1,309 ± 120 after

160 mg., and 1,392 ± 137 after 320 mg. of meralluride. This illustrates a significant relationship of response in sodium excretion to the dose until the 160 mg. dose level is passed after which no additional response is observed. The excretion rates of potassium and bicarbonate were not altered; chloride excretion paralleled the sodium excretion. The intravenous administration of chlorothiazide produced a sodium excretion of 397 ± 36 μEq per minute after 500 mg., 815 ± 82 after 2,000 mg., and 892 ± 96 after 4,000 mg. of the drug.

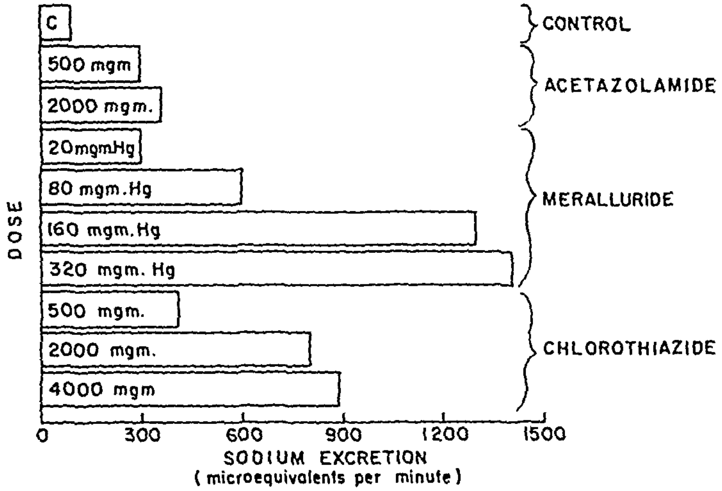

Fig. 2.—The effect of 3 diuretics given intravenously on sodium excretion. The comparative bar graphs illustrate a distinct dependence of sodium excretion upon the dose of meralluride and chlorothiazide but the last two doses show no significant differences.

This illustrates, as in the case of meralluride, that there is a significant relationship of sodium response to the dose of the drug until the 2,000 mg. dose is exceeded, beyond that further increase in dosage is not accompanied by an increased sodium excretory rate. The excretion rate of potassium and bicarbonate was increased to about 35 per cent of the sodium rate; chloride excretion paralleled the sodium excretion.

C. The Effect of Combinations of Meralluride and Acetazoleamide on the Excretion of Sodium and Other Electrolytes (Fig. 3).—After the administration of meralluride, 160 mg. mercury equivalent (4 ml.), as a priming dose and 160 mg. as a sustaining infusion, there was an increase in sodium excretion from a control value of 110 μEq per minute to 1,360 ± 170. As the sustaining infusion of saline containing the meralluride was continued and while the sodium excretory rate was maximum and stable (at the end of 2 hours) the intravenous administration of 500 mg. acetazoleamide, prime only, produced a further increase in sodium excretion to 1,920 ± 310 μEq per minute. Chloride excretion after meralluride paralleled sodium excretion while potassium and bicarbonate were not altered. After acetazoleamide was added, the chloride excretion rate was reduced to only 50 per cent of the sodium rate while bicarbonate and potassium excretion rates increased to 50 per cent of the sodium rate.

In a subsequent study the administration of acetazoleamide 2 000 mg prime and 2 000 mg sustaining produced an increase in sodium excretion to 345 ± 52 μEq per minute which was maximum and stable at the end of 2 hours At this point a priming dose of meralluride 20 mg (0 5 ml) produced a further increase to 722 ± 119 μEq per minute The excretion rate of bicarbonate and potassium after acetazoleamide was increased to about 90 per cent of the sodium rate while chloride excretion was depressed below the control rate After meralluride was added the chloride excretion rate increased to 80 per cent of the sodium rate while bicarbonate and potassium excretion rates were not altered from the original acetazoleamide effect

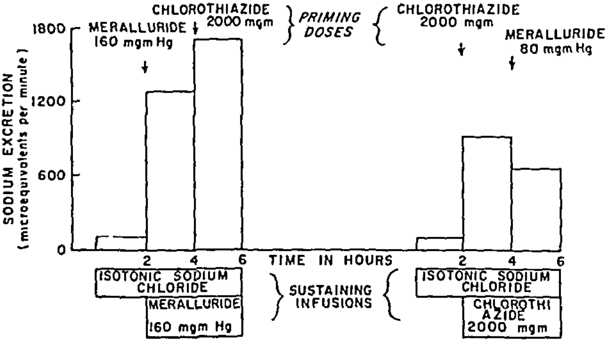

Fig 3—Th ralluride and acetazoleamide on sodium excre-
tion illustrating action Even with a maximum response from
either meralluride nistration of the other drug produces an addi-
tional increase activation of a second receptor site

D The Effect of Combinations of Meralluride and Chlorothiazide on the Excretion of Sodium and Other Electrolytes (Fig 4)—After the administration of 160 mg mercury equivalent of meralluride (4 ml) as prime and 160 mg as sustaining, the sodium excretion increased to 1,360 ± 170 μEq per minute which was maximum and stable at the end of 2 hours At this point a priming dose of 2,000 mg chlorothiazide produced a further increase in sodium excretion to 1,732 + 158 μEq per minute The chloride excretion rate was increased after meralluride to parallel the sodium rate while bicarbonate and potassium were not altered After chlorothiazide was added, bicarbonate and potassium excretion rates were increased to about 35 per cent of the sodium rate while the chloride excretion rate was only 60 per cent of the sodium rate

In a subsequent study the order was reversed so that a 2,000 mg priming dose and a 2,000 mg sustaining dose of chlorothiazide produced a sodium excretion of 860 ± 92 μEq per minute which was maximum and stable at the end of 2 hours At this point a priming dose of meralluride, 80 mg mercury equivalent (2 ml), produced no further increase in the rate of sodium excretion (780 ± 82 μEq per minute) After the chlorothiazide administration the

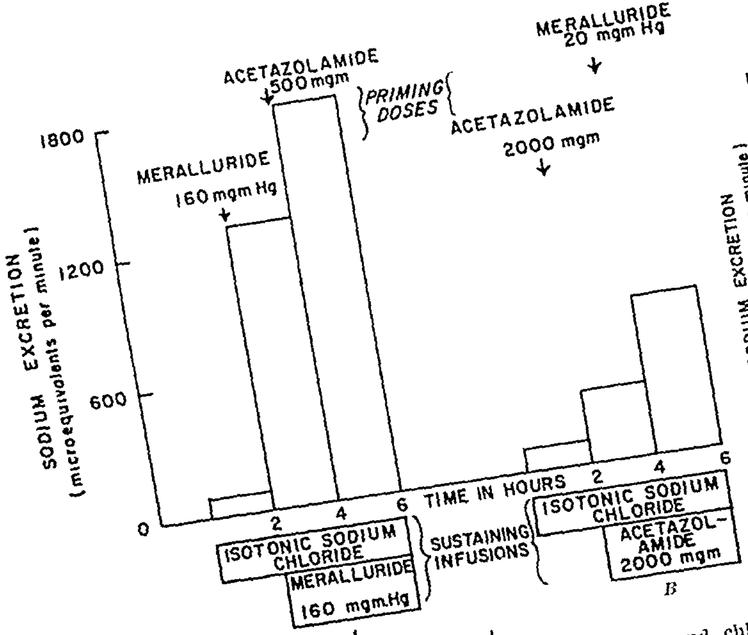

Fig 1.

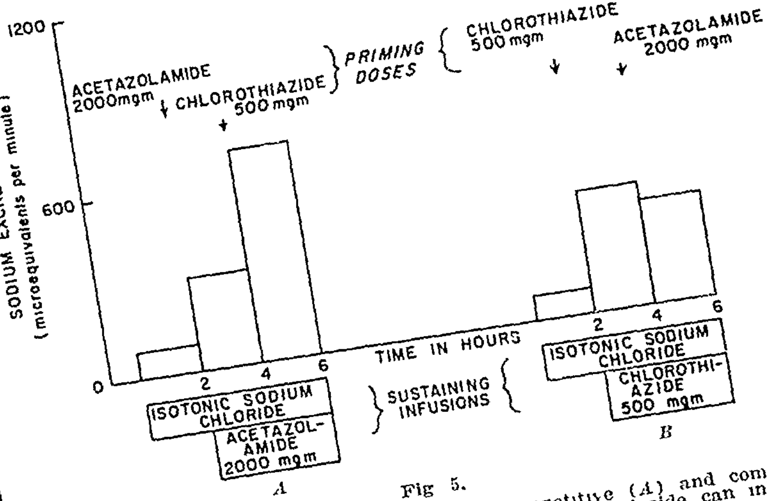

Fig 5.

Fig 1.—The effect of combinations of meralluride and chlorothiazide on sodium excretion illustrating noncompetitive (*A*) and competitive (*B*) mechanisms The additional sodium excretion from chlorothiazide cannot be inhibited by meralluride but chlorothiazide can inhibit an additional sodium response from meralluride.

Fig 5.—The effect of combinations of acetazoleamide and chlorothiazide on sodium excretion illustrating noncompetitive (*A*) and competitive (*B*) mechanisms The additional sodium excretion from chlorothiazide cannot be inhibited by acetazoleamide, but chlorothiazide can inhibit an addition sodium response from acetazoleamide

excretion rate of potassium and bicarbonate was increased to about 35 per cent of the sodium rate while chloride excretion paralleled the sodium excretion. When meralluride was added no significant changes in potassium, chloride or bicarbonate were observed.

E The Effect of Combinations of Acetazoleamide and Chlorothiazide on Electrolyte Excretion (Fig 5)—The administration of 2,000 mg of acetazoleamide as a prime and 2,000 mg as a sustaining dose produced a sodium excretion of 345 ± 52 μEq per minute which was maximum and stable at the end of 2 hours. At this point, a 500 mg priming dose of chlorothiazide produced an additional increase in sodium excretion to 720 ± 88 μEq per minute.

In a subsequent study the order was reversed so that 500 mg of both a priming and a sustaining dose of chlorothiazide, produced a sodium excretion of 442 ± 65 μEq per minute which was maximum and stable at the end of 2 hours. At this point, a 2,000 mg priming dose of acetazoleamide produced no further increase in sodium excretion (380 ± 51 μEq per minute).

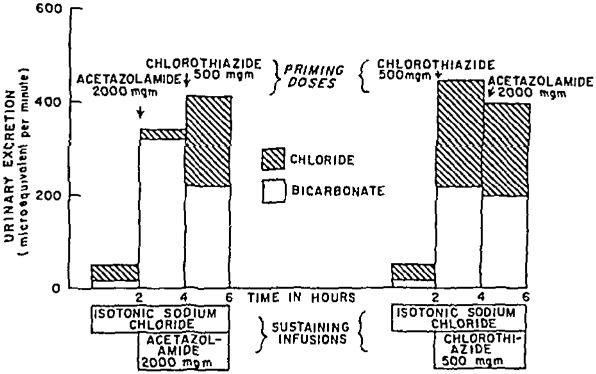

Fig 6.—The effect of combinations of acetazoleamide and chlorothiazide on chloride and bicarbonate excretion. Chlorothiazide decreases the bicarbonate response produced by acetazoleamide but augments chloride.

To further elaborate on this situation a single typical study of the excretion of bicarbonate and chloride under the same conditions is presented in Fig 6. After the priming and sustaining doses of acetazoleamide, bicarbonate excretion rate increases to 320 while the chloride excretion rate is 20 μEq per minute (control bicarbonate was less than 10 and chloride less than 40). After chlorothiazide was added as a prime (in the same study) bicarbonate excretion dropped to 220 and chloride increased to 190 μEq per minute. In a subsequent study, when chlorothiazide was administered first, bicarbonate excretion increased to 220 and chloride to 230 μEq per minute. However,

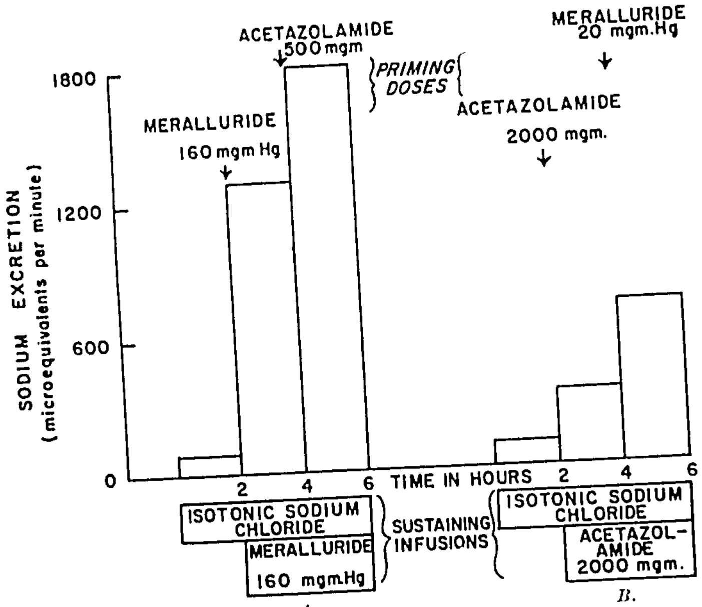

Fig. 4.

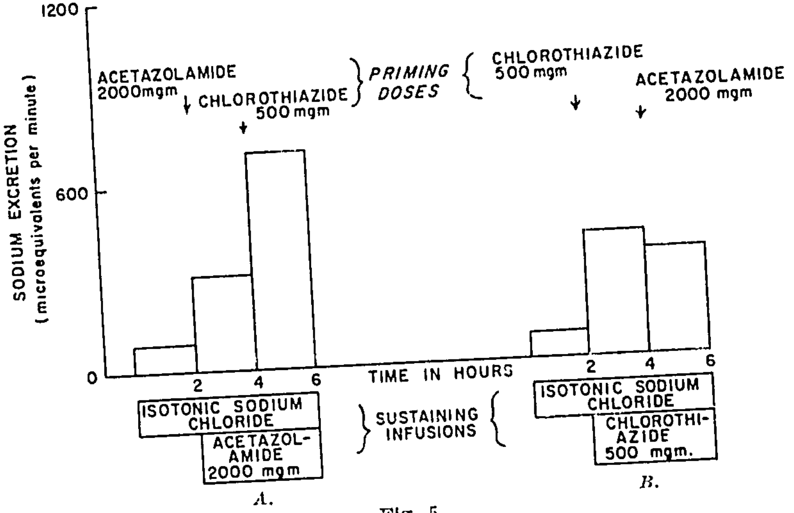

Fig. 5.

Fig. 4.—The effect of combinations of meralluride and chlorothiazide on sodium excretion illustrating noncompetitive (*A*) and competitive (*B*) mechanisms. The additional sodium excretion from chlorothiazide cannot be inhibited by meralluride but chlorothiazide can inhibit an additional sodium response from meralluride.

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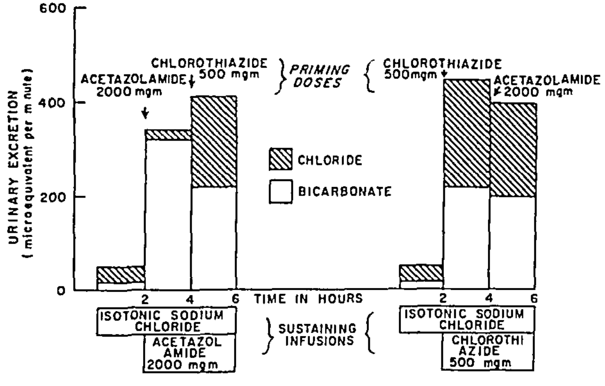
In a subsequent study the order was reversed so that 500 mg. of both a priming and a sustaining dose of chlorothiazide produced a sodium excretion of 442 ± 65 μEq per minute which was maximum and stable at the end of 2 hours. At this point a 2,000 mg. priming dose of acetazoleamide produced no further increase in sodium excretion (380 + 51 μEq per minute).



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To further elaborate on this situation a single typical study of the excretion of bicarbonate and chloride under the same conditions is presented in Fig. 6. After the priming and sustaining doses of acetazoleamide, bicarbonate excretion rate increases to 320 while the chloride excretion rate is 20 μEq per minute (control bicarbonate was less than 10 and chloride less than 40). After chlorothiazide was added as a prime (in the same study) bicarbonate excretion dropped to 220 and chloride increased to 190 μEq per minute. In a subsequent study, when chlorothiazide was administered first, bicarbonate excretion increased to 220 and chloride to 230 μEq per minute. However, after acetazoleamide was added as a prime (in the same study) bicarbonate excretion rate was then 200 and chloride 200 μEq per minute. Potassium excretion (not graphed), in general, paralleled the bicarbonate excretion.

F. The Effect of Combinations of Meralluride, Acetazoleamide, and Chlorothiazide on the Excretion of Sodium and Other Electrolytes (Fig. 7).—The administration of meralluride, 160 mg. mercury equivalent (4 ml.), as a prime and 160 mg. as a sustainer produced a sodium excretion of 1,360 ± 170 μEq per minute which was maximum and stable at the end of 2 hours. At this point, 2,000 mg. acetazoleamide, both as a priming and a sustaining dose, produced an additional increase in sodium excretion to 1,742 ± 182 μEq per minute which was maximum and stable at the end of the second 2-hour period. At this point, a 500 mg. priming dose of chlorothiazide produced a third increase in sodium excretion to 2,249 ± 240 μEq per minute which was maximum and stable at the end of the third 2-hour period.

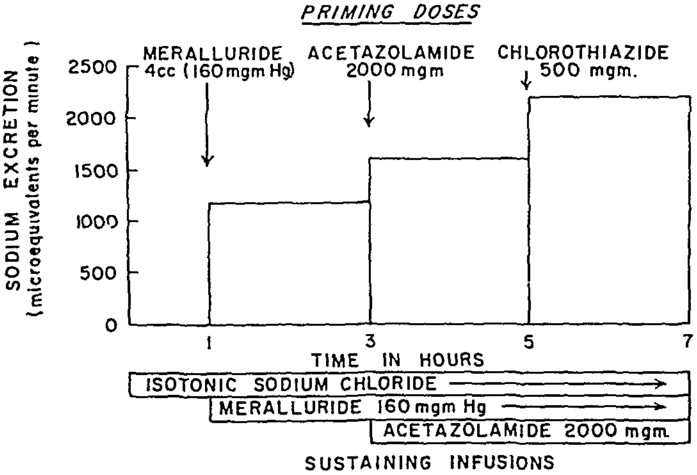

Fig. 7.—The effect of a combination of meralluride, acetazoleamide, and chlorothiazide on sodium excretion illustrating noncompetitive mechanisms. In spite of maximum sodium responses from meralluride and acetazoleamide, a small dose of chlorothiazide produces a third increase in sodium excretion.

The excretion of potassium was similar to that observed in previous studies and, in general, paralleled bicarbonate excretion. The excretion of bicarbonate was similar to that in previous studies and generally increased after acetazoleamide (35 per cent of the sodium rate) and declined after chlorothiazide was added to only 25 per cent of the sodium rate. Chloride excretion was also similar to that in previous studies and, in general, increased after meralluride to a degree similar to that of sodium. After acetazoleamide was added, chloride excretion rate decreased to about 50 per cent of the sodium rate. However, after chlorothiazide was added the chloride excretion rate once more increased to about 70 per cent of the sodium rate.

G. Renal Hemodynamic Effects.—Contemporary observations of glomerular filtration rate (inulin clearance) and renal plasma flow (para-aminohippurate clearance) revealed no significant changes (data not presented).

DISCUSSION

The synthesis and exploration of varying chemical compounds with diuretic action has encouraged speculation concerning their mechanisms of action. Admittedly, this area of man's knowledge is woefully inadequate due to insufficient techniques for study. Histochemical studies have furnished certain bits of information implicating specific enzyme system inhibition by mercurial diuretic therapy. Also, the ability of sulfonamides and other agents to inhibit the carbonic anhydrase system is fairly well documented by in vitro studies. These drug effects inhibit sources of energy for the activation of the ion exchange mechanism for sodium reabsorption. Although this information is valuable, it is incomplete and, furthermore, does not necessarily describe the specific electrolyte excretion effects to be expected from specific enzymatic inhibition.

The purpose of this study therefore, is to present indirect evidence concerning the mechanisms of action of 3 groups of diuretics. If one proceeds with the hypothesis that the primary action of diuretics is to inhibit the renal tubular reabsorption of sodium, changes in urinary sodium may afford indirect evidence of diuretic action. Furthermore, the relationships of the urinary excretion of various other electrolytes, especially potassium, chloride, and bicarbonate, under conditions of diuretic therapy may afford additional mechanistic information. The concept to be tested involves the "saturation" of the hypothetical receptor site, or the inhibition of one renal transport mechanism by the first drug to be followed by the second drug administration, and the observation of subsequent changes in sodium excretion. Dosages of the "second" drugs were selected to avoid the factor of potency (see Materials and Methods). If the second (or third) drug effected an additional increase in sodium excretion, one might speculate that a second (or third) receptor site or transport mechanism had been affected. If the second (or third) drug failed to effect an additional increase in sodium excretion, one might speculate that the receptor site or transport mechanism of the first drug had been "saturated" and that the second drug had no other site (or mechanism) through which to act and that their mechanisms were "competitive."

The results of this study suggest that the mechanisms of action (? enzyme system inhibition) of merallunide and acetazoleamide are different or noncompetitive. This conclusion is based on the significant increase in sodium excretion following the administration of acetazoleamide *after* a maximum excretory rate of sodium excretion has been produced by merallunide as a priming injection and during the continued administration of merallunide as a sustaining infusion. Additional evidence is furnished by the results of the study in which the order of drugs is reversed. A similar effect is observed when acetazoleamide is administered as a priming injection and sustained throughout the study. Although an equipotent (or less) dose of merallunide is then injected, there is still an increase in sodium excretion. In other words, massive doses of either acetazoleamide or merallunide cannot "block" the effect of subsequent doses of the other drug (meralluride or acetazoleamide) on the excretion of sodium suggesting that a second receptor site has been acted upon, a second renal transport mechanism inhibited.

A slightly different conclusion must be drawn concerning the relationships of meralluride and chlorothiazide. Since chlorothiazide can further increase the excretion of sodium even after a maximal effect has been produced by meralluride, another mechanism or receptor site appears to be involved. The converse is not true, however. After a maximum effect has been produced by chlorothiazide, the addition of meralluride cannot further augment sodium excretion. This suggests that chlorothiazide and meralluride share a single mechanism of action or receptor site (for transport inhibition) but, also, that chlorothiazide can augment sodium excretion by inhibition of another transport mechanism. Meralluride apparently can augment sodium excretion by inhibition of only a single sodium reabsorptive transport mechanism. In other words, chlorothiazide can "block" the effect of subsequent doses of meralluride but meralluride cannot "block" the effect of subsequent doses of chlorothiazide on the excretion of sodium.

The relationships of acetazoleamide and chlorothiazide reflect a somewhat similar situation. Although chlorothiazide can further augment sodium excretion even after a maximum effect has been produced by acetazoleamide, the converse is not true. This suggests that these two drugs share one mechanism of action or receptor site but that chlorothiazide can exert its effects through yet another mechanism. In other words, chlorothiazide can "block" the effect of subsequent doses of acetazoleamide but acetazoleamide cannot "block" the effect of subsequent doses of chlorothiazide on the excretion of sodium.

That chlorothiazide does not necessarily use the "acetazoleamide mechanism" when blocked by meralluride and the "meralluride mechanism" when blocked by acetazoleamide, is indicated by the results of the final segment of this study. Although both transport mechanisms have been inhibited by the prior priming doses and continued sustaining doses of both meralluride and acetazoleamide, the subsequent injection of even a small dose of chlorothiazide evokes a further increase in sodium excretion. This suggests that chlorothiazide activity cannot be "blocked" by meralluride or acetazoleamide (singly or in combination) although it is capable of "blocking" both of them. This may be considered indirect evidence that chlorothiazide produces its effect in inhibiting the renal tubular reabsorption of sodium by a third mechanism of action which is noncompetitive with the mechanisms of action of meralluride and acetazoleamide. That this may indicate a greater range of activity of enzyme binding throughout the nephron by chlorothiazide must be considered, but the data at hand do not permit us to differentiate this point. It must be emphasized that these changes were observed without contemporary change in renal hemodynamics (glomerular filtration rate and renal plasma flow).

The relationships of other urinary electrolytes, such as potassium, chloride, and bicarbonate, lend further support to these conclusions Detailed description of these changes have been omitted from the discussion since the primary consideration of the moment is that of diuretic action which we consider to be primarily natriuretic

SUMMARY

Indirect evidence concerning the mechanisms of action of meralluride, acetazoleamide, and chlorothiazide as diuretics has been presented in data consisting of the urinary excretion of sodium, potassium, chloride, and bicarbonate *The primary action of these diuretics is the augmentation of the renal excretion of sodium*

The data suggest that the mechanisms of action of meralluride and acetazoleamide are noncompetitive and different, i e, different "receptor sites" are acted upon or different renal transport mechanisms for the reabsorption of sodium are inhibited

The mechanism of action of meralluride is competitive with that of chlorothiazide (the same). Yet, chlorothiazide apparently can act through another mechanism which is noncompetitive with meralluride but another mechanism is apparently not available to meralluride

The mechanism of action of acetazoleamide is competitive with that of chlorothiazide (the same). Yet, chlorothiazide apparently can act through another mechanism which is noncompetitive with acetazoleamide

The third mechanism of action as a diuretic exemplified by chlorothiazide cannot be inhibited by the concurrent administration of meralluride and acetazoleamide, i e, the receptor site (for the inhibition of renal reabsorption of sodium) of chlorothiazide cannot be "saturated" by meralluride or acetazoleamide.

This additional mechanism of action of chlorothiazide may imply involvement of another enzyme system or it may imply a greater range of activity of chlorothiazide throughout the nephron

We express our sincere gratitude to Drs Robert Pitts, Karl Beyer, and Carroll Handley, for their review of this manuscript and for helpful comments

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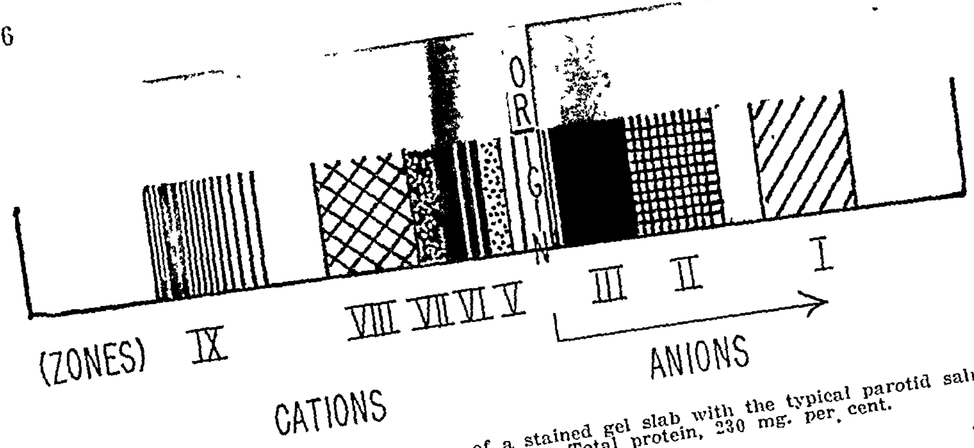

Fig. 1.—Photograph and drawn diagram of a stained gel slab with the typical parotid saliva protein component distribution shown. Total protein, 230 mg. per cent.

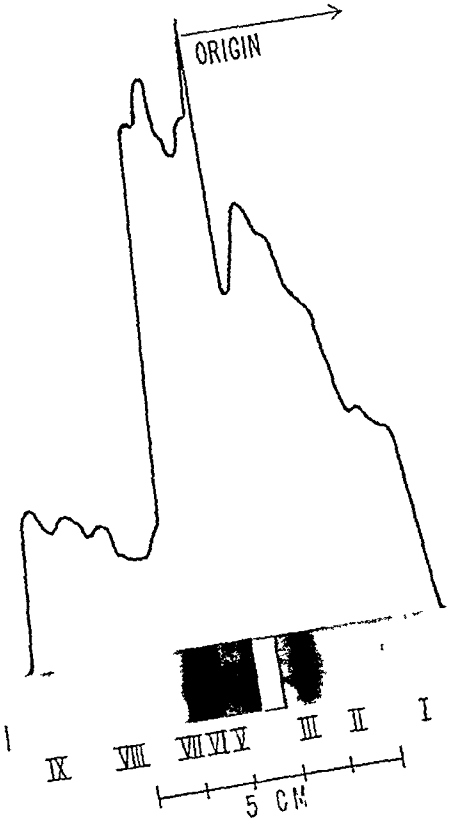

Fig. 2.—Photograph of a stained gel slab showing parotid protein distribution with an accompanying concentration curve approximated by means of a scan of a standardized photograph of the gel slab in an Aminco JLC photodensitometer.

Fig 3 shows the result of an experiment in which a parotid protein and blood serum sample were inserted in the same gel slab and subjected to electrophoresis Parotid components I and III exhibited migrations similar to serum albumin and beta globulin respectively However, information gained in such an experiment would serve merely as a guide to isolation and preparative procedures leading to absolute comparative identification of parotid components I and III

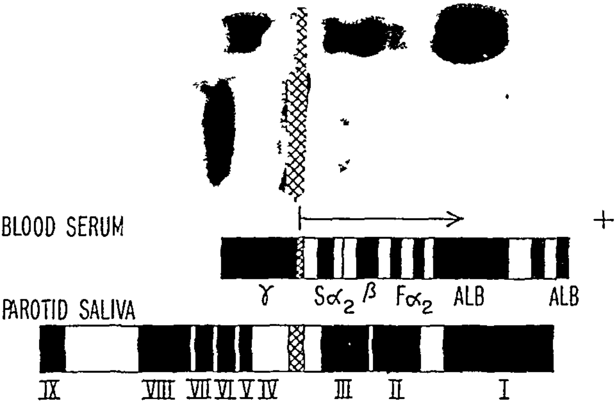

Fig 3 —Photograph and explanatory diagram of a gel slab in which parotid saliva protein and blood serum were subjected to electrophoresis concomitantly

DISCUSSION

Control of parotid amylase action on the starch gel support was accomplished in an acceptable fashion by employing a relatively chloride free protein sample as a result of the acetone precipitation method, and electrophoresis runs at low temperatures with increased potential gradients Some amylase activity usually occurred immediately off the point of sample insertion toward the cathode It was of such an order, however, as to have no deleterious effect on the supporting medium and, in turn, on the migration of the faster moving cationic components

Changes in the physical properties of the starch gels after staining and washing prevented the accurate measurement of migration constants And the indirect method of obtaining concentration curves from standardized photographs of the stained gels was considered of little quantitative value

Among the advantages of the method are (1) simplicity and convenience, (2) reduced volumes of native parotid secretion (protein) required, (3) possibility of characterization of distinct components by specific color reactions or application of radioactive isotopes and (4) ability to isolate various protein components for further biologic classification

SUMMARY

A method for separation of parotid saliva proteins by zone electrophoresis in starch gels has been presented. It was found simple, convenient, and free from the great technical requirements of classic boundary electrophoresis.

A study of 10 samples of parotid saliva subjected to electrophoresis by the present method revealed reproducible protein migration patterns. Nine to twelve protein components were regularly resolved. This finding was in good agreement with those reported in which boundary electrophoresis was employed. The majority of components migrated as cations at pH 8.4.

Two parotid protein components were observed to migrate in the fashion of blood serum proteins subjected to similar electrophoretic conditions. Absolute identification of these parotid components on a comparative basis awaits further investigation.

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RELATIONSHIP OF EQUILIBRIUM OF DISTRIBUTION, BIOLOGIC DECAY RATES, AND SPACE AND MASS OF H^3, Cl^{36}, AND Rb^{86} OBSERVED SIMULTANEOUSLY IN A COMFORTABLE AND IN A HOT AND HUMID ENVIRONMENT IN CONTROL SUBJECTS AND IN PATIENTS WITH CONGESTIVE HEART FAILURE

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IN ORDER to study the demands placed on the vascular system by thermal regulation in a hot and humid environment, daily clinical studies were made of unselected patients admitted to adjoining wards at the Charity Hospital, one was maintained at comfortable conditions with air conditioning and the other was exposed to the hot and humid environment of New Orleans in midsummer. In most instances even though the patients' impression and ordinary clinical observations readily convinced the clinician of the marked benefits of the comfortable environment such impressions were not substantiated by differences in the pulse rates, character and rate of respiration, blood pressure, body temperature, and other physiologic phenomena between the two groups. Since a hot and humid environment profoundly influences sweating and water and electrolyte intake as well as other phenomena concerned with thermal regulation, the details of certain aspects of electrolyte and water metabolism were investigated by tracer methods already in use in this laboratory to learn the influence of these two types of environments on sick people. Control subjects without disturbance in water and electrolyte metabolism were observed along with patients with chronic congestive heart failure.

Tritium, Rb^{86}, and Cl^{36} were employed simultaneously as tracers along with determinations of stable electrolytes under controlled metabolic conditions. In addition, the experiments were also designed to elucidate water and electrolyte metabolism in man by simultaneous observations of the 3 tracers and nontracer elements.

METHODS AND MATERIALS

Ten Negro women (see Table I for clinical data) were studied under controlled metabolic conditions in the wards of Charity Hospital during the hot and humid period of July 9 to Aug. 3, 1956. Five subjects were injected simultaneously with Cl^{36} (as

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NaCl), Rb^{86} (as $Rb^{86}Cl$), and H^3 (as H^3OH) in doses shown in Table I. Metabolic data included intake and output of K^{39}, Cl^{35}, Na^{23}, and water. Previous studies had shown the loss of chloride, sodium, and potassium in normal stools to be negligible,[1, 2] and there were no instances of diarrhea among the subjects in this study. Body weight was recorded each morning for all subjects when the clinical state permitted.

TABLE I. CLINICAL DATA ON THE FIVE SUBJECTS STUDIED

PATIENT NUMBER	AGE (YR.)	DIAGNOSIS	DOSE OF ISOTOPES Cl^{36} (MC.)	Rb^{86} (MC.)	H^3 (MC.)	TREATMENT
1	46	Control, convalescent, upper respiratory infection	0.026	0.631	3.749	None
2	50	Control, tuberculous arthritis	0.027	0.663	4.104	Streptomycin, sodium para-amino salicylate, body and leg cast
3	52	R.H.D., C.H.F.	0.020	0.481	2.993	Digitalis mercurial diuretics
4	52	A.S.H.D., H.H.D., C.H.F.	0.027	0.662	3.780	Digitalis mercurial diuretics
5	65	A.S.H.D., C.H.F., empyema, pneumothorax	0.026	0.642	3.852	Digitalis penicillin thoracentesis

Key to abbreviations: A.S.H.D. = arteriosclerotic heart disease; H.H.D. = hypertensive heart disease; R.H.D = rheumatic heart disease; C.H.F. = congestive heart failure

Samples of blood were collected daily at 8:00 A.M. prior to eating; one aliquot was collected under oil for studies of the plasma and another aliquot was used for studies of whole blood. Studies on the plasma included measurements of Cl^{36}, Rb^{86}, H^3, Cl^{35}, Na^{23}, K^{39}, creatinine, and osmolarity. Studies on the whole blood included measurements of Cl^{36}, Rb^{86}, H^3, Na^{23}, K^{39}, and hematocrit. The concentrations of Cl^{36}, Rb^{86}, and H^3 in the erythrocytes were computed by the following equation:

$$C_R = \frac{C_{WB} - (1 - H)\ C_P}{H}$$

where C_R = the concentration of the substance in erythrocytes; C_{WB} = the concentration of the substance in whole blood; C_P = the concentration of the substance in plasma; and H = the hematocrit.

The urine was collected under toluene from 8:00 A.M. to 8:00 A.M. of the following day. The volume of urine was recorded along with specific gravity, pH, osmolarity, and Na^{23}, Cl^{35}, Cl^{36}, Rb^{86}, K^{39}, H^3, and creatinine.

All samples of whole blood were laked by freezing and thawing before radiometric or chemical analyses were made. Sodium and potassium content of whole blood, serum, and urine was determined by flame photometry, chloride by the method of Schales and Schales,[3] osmolarity of serum and urine by the Fiske osmometer, and creatinine by the method of Haugen.[4]

The concentrations of Rb^{86} and Cl^{36} were determined by the method previously described.[5] Separation of the two isotopes in the same sample was achieved by counting a few hours after collection of the samples and then recounting 200 days later after the Rb^{86} had decayed. Radioassay for tritium was preceded by distilling the [illegible] whole blood, urine and serum at 90° to 100° C. The distillate contained no Cl^{36}. Radioassay of the tritium in this water distillate was done with a Packard Tri-Carb liquid scintillation counter.*

The subjects were divided into equal groups: one group was observed in the comfortable air-conditioned ward (mean temperature 74.7° F, range 70.1° to 80.2° F and a mean relative humidity of 73.4 per cent with extremes of 90 per cent and 60 per cent), and the other group was in a subtropical summer environment of an ordinary ward in July. The average daily temperature recorded twice daily on the ward was 86.8° F with a relative humidity of 62.0 per cent. For the period of observation the Weather Bureau reports revealed a mean temperature of 83.4° F, range 91° F to 69° F, and a mean relative humidity of 80.0 per cent, range 98 per cent to 47 per cent. The maximum daily temperature was 90.0° F or greater on 21 of the 26 days of observation. At the end of approximately 2 weeks the subjects were changed to the other environment.

RESULTS

For convenience of presentation the results summarized in Figs. 1 to 6 and Tables II and III are divided into 3 sections: equilibrium of distribution, biologic decay rates, and measurements of space and mass.

Equilibrium of Distribution.—Previous experiments[8] indicated that it was not possible to determine in intact man the moment at which equilibrium of distribution of radioelements is established. Since only a limited number of isotopic compartments of the body could be sampled simultaneously, equilibrium of distribution of Cl^{36}, Rb^{86}, and H^3 was estimated from the following: (a) the constancy of slope of the concentration-time course curves of the tracers, (b) the constancy of the magnitudes of the calculated space and mass of the nontracer elements, and (c) the specific activity of more than one compartment, namely, plasma and erythrocytes for Rb^{86}. The values noted below were derived from these 3 methods.

Radiochloride. Equilibrium of distribution of Cl^{36} probably did not occur until at least 3 to 4 days following injection of the tracer (Figs. 1, 2, 3, 5, and 6). There were no detectable differences between comparable subjects at different environmental temperatures; however, the presence of congestive heart failure tended to delay the development of equilibrium of distribution of Cl^{36} in both environments.

Radiorubidium. It is probable that equilibrium of distribution of Rb^{86} was not established at any time during these observations and certainly not for the initial 7 to 11 days. This was supported by the fact that the concentration of Rb^{86} in the erythrocytes was increasing while that in the plasma was declining for the first 7 to 11 days after the injection of the tracer. No effect of environment or congestive heart failure could be detected in these concentration-time course curves of Rb^{86}.

Tritium. The concentration-time course of tritium in plasma and erythrocytes showed that equilibrium of distribution was established within 24 hours. Since equilibrium occurred in all cases before the first samples of blood were collected, no effect of environment or congestive heart failure could be noted.

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*The authors wish to express their appreciation to Drs. Leon Jacobsen and George Okita and associates, University of Chicago School of Medicine, for the use of their laboratory facilities for counting the tritium samples.

Comment: The subjects were allowed to drink water as desired and eat a regular diet of known electrolyte content. The daily intake of the nontracer material was sufficient to exceed the increased requirements produced by the stress of the hot and humid environment so that the influence of the environment was not made evident in the rate of development of equilibrium of distribution of Cl^{36}, Rb^{86}, and H^3. Had the intake of nontracer materials been inadequate to meet these increased demands with resultant functional sequestration of electrolyte compartments in the body, then impairment of the development of a state of equilibrium of distribution might have been expected. Congestive heart failure, on the other hand, with impairment of the steady state of metabolism for Cl^{36}, Rb^{86}, and H^3 might be expected to interfere with equilibrium of distribution. This was evident for Cl^{36} only.

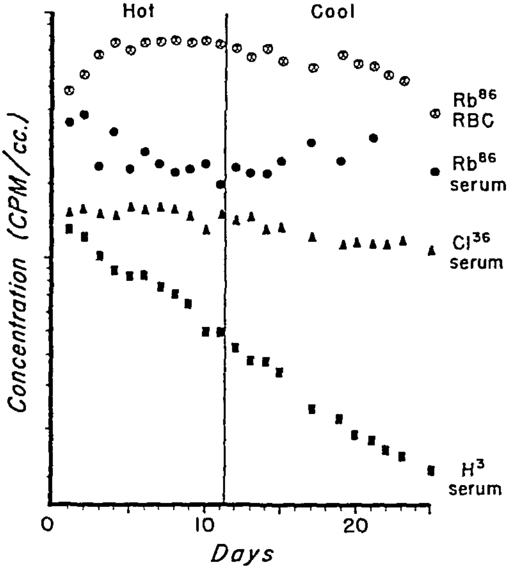

Fig. 1.—Biologic decay rates of Rb^{86}, Cl^{36}, and H^3 in Subject 5 with congestive heart failure. The concentration-time course curves are shown for Rb^{86} in the erythrocytes and serum, Cl^{36} in the serum, and H^3 in serum in that order from above downward. The ordinate is logarithmic in this and subsequent figures. The numerical value of concentration of these radioisotopes is not shown since only the rate of decline in concentration is considered. The vertical line indicates the day of study on which the environment was changed.

Since Rb^{86} entered the potassium metabolic pool in the body, the isotope became predominantly intracellular. Therefore, unless the hot and humid environment or congestive heart failure caused sufficient alterations in the rate of the metabolic processes of K^{39} in cells, the environmental conditions would not be expected to modify the rate with which equilibrium of distribution of Rb^{86} was approached. The slow rate of exchange of some of the cellular compartments is reflected in the time required for the concentration of the Rb^{86} to reach a maximum level in the erythrocytes. The importance of the slow rate of uptake as well as the lag in release of intracellular electrolyte back into the plasma in estimating the time of equilibrium of distribution has been previously indicated.[8, 11] As long as the time course curves of "specific activity" in different compartments are not parallel, equilibrium of distribution cannot exist. Furthermore, lags in exchange of the tracer result in inconstant calculated values for space and mass with time. The dependence of the concentration of Rb^{86} in the plasma on the "feed back" from cellular compartments in general is indicated by the parallel decline in concentration of Rb^{86} in the plasma and erythrocytes in the latter part of these experiments (Figs. 1 to 3).

Tritium was studied in two compartments, plasma and erythrocytes. There was no difference in concentration of H^3 in these two compartments by the time the first sample of blood was withdrawn, i.e., 20 to 22 hours after injection. The concentration time course of H^3 in plasma revealed a constant rate of decline from the first measurement throughout the duration of these experiments. Since there were large unmeasured extraurinary losses of water via skin, respiratory tract, and stool, the values of H^3 space show a continuing expansion. Therefore, a time course curve of space and mass of H_2O was not satisfactory for estimating the time at which equilibrium of distribution developed.

Numerous studies of the time required for equilibrium of distribution of deuterium and tritium to develop in animals have been reported.[12, 19] There is considerable evidence that equilibrium of distribution of H^3 develops rapidly in man, although very little published data are based upon experiments extending as long as 24 hours after injection of the tracer.[20, 29] The rapid rate at which equilibrium of distribution of the isotopes of hydrogen develops indicates its distribution is primarily with the hydrogen of the water of the body. The exchange is slow with the other hydrogen atoms of the body.[13, 17, 30, 33] In chemical reactions involving hydrogen such as in enzymatic systems, there is preference for ordinary hydrogen over its two heavier isotopes.[13, 17, 30, 32, 34, 35] The amount of heavy isotope involved in such chemical linkages is a very small portion of a single injection, probably not amounting to over 1.5 to 2 per cent of the total dose. Since such a small amount is involved in slow exchanges, in both incorporation and in release from relatively stable chemical bonds, the effects of the isotopes are not evident in tracer studies of this type.

Biologic Decay Rates.—The biologic decay rates were determined as previously described.[6, 8] $C_{1/2}$ values were obtained for 5 subjects and $U_{1/2}$ was determined for 4 subjects. These parameters have the following connotations:

$C_{1/2}$ represents the length of time required for the concentration of the tracer to decline to one half concentration in the compartment concerned at any time following equilibrium of distribution. $C_{1/2}$ values in these experiments were determined for the plasma compartment for Cl^{36}, Rb^{86}, and H^3 and in the erythrocyte compartment for Rb^{86}. $U_{1/2}$ represents the length of time required to recover one half the administered isotope in the urine.

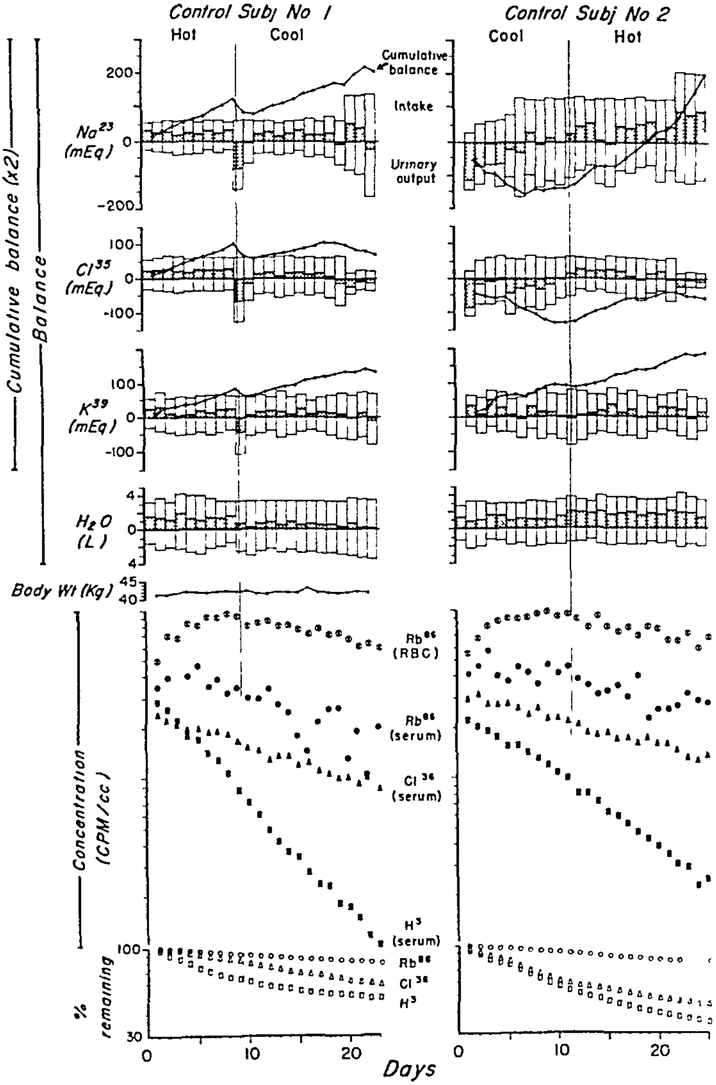

Fig. 2.—Biologic decay rates of Rb^{86}, Cl^{36}, and H^3 and metabolic balance data on 2 control subjects. The concentration-time course curves are presented as in Fig. 1. In the lower portion of the graph the per cent remaining in the body (not recovered in the urine) is expressed as a percentage of the injected dose of radioisotopes. In the portion of the graph showing electrolyte and water balance intake is indicated by columns above the base line, urinary output by columns below the base line, and balance by the solid columns. When balance was positive, the solid column is above the base line. The ordinate value for the cumulative balance is twice the values for the other balance data.

The average values obtained for the 3 parameters are summarized in Table II and in Figs. 1, 2, and 3.

TABLE II. THE INFLUENCE OF ENVIRONMENT ON BIOLOGIC DECAY RATES OF Rb^{86}, Cl^{36}, AND H^{3}

BIOLOGIC DECAY RATES (DAYS) C ½

SUBJECT	Cl^{36}			Rb^{86}					H^{3}		
				SERUM			ERYTHROCYTES				
	ENTIRE STUDY	HOT	COLD	ENTIRE STUDY	HOT	COLD	DESCENDING CURVE ONLY*	ENTIRE STUDY	ENTIRE STUDY	HOT	COLD
1	14.9	15.5	14.6	28.0	238.1	18.4	26.8	86.0	4.6	4.5	4.6
2	18.8	20.2	17.1	27.2	18.3	84.9	22.0	66.3	7.5	7.0	8.4
3	22.8	17.9	25.9	57.2		9.6	42.9	82.0	7.0	8.1	6.6
4	28.0	27.5	28.8	37.8	175.9	18.0	22.1	52.7	6.6	5.2	9.1
5	46.8	382.9	28.8	16.9	61		16.8	36.1	7.2	7.1	7.5
Mean	26.2	92.8	23.0	33.4	134.6	54.2	20.1	64.8	6.6	6.4	7.2

U ½

SUBJECT	Cl^{36}			Rb^{86}			H^{3}		
	ENTIRE STUDY	HOT	COLD	ENTIRE STUDY	HOT	COLD	ENTIRE STUDY	HOT	COLD
1	31.5	24.2	29.9	74.9	66.8	81.2	22.1	14.2	28.6
2	21.3	32.1	14.9	68.7	78.0	59.5	16.5	22.6	12.3
3	96.7†	99.4†	88.6†	58.2†	60.7†	57.4†	20.6†	29.9†	18.7†
	17.6			53.1			17.4		
4	46.0†	54.8†	38.9†		122.9†	87.1†		61.2†	23.7†
	39.2	39.5		101.9	117.5		34.2	49.8	
5	‡								
Mean	48.9	55.2	43.1	75.9	82.1	71.3	23.6	22.0	23.3

SUBJECT	PER CENT RECOVERED IN URINE		
	Cl^{36}	Rb^{86}	H^{3}
1	39.8	19.4	50.0
2	55.8	21.0	65.1
3	59.1	24.0	56.7
4	36.0	15.8	40.0
Mean	47.7	20.0	53.0

*Calculated from that part of the concentration time course curve which followed the peak.

†This signifies figures calculated without mercury.

‡Incomplete urine collections.

Radiochloride. The $C_{1/2}$ and $U_{1/2}$ values for chloride were less for the control subjects than for the subjects with congestive heart failure. There was no definite effect of the two environments on these parameters for Cl^{36} in any of the subjects studied. Most of the chloride excreted by Subject 3 was in response to mercurial diuretics so that effects of the environment could have been masked (Fig. 3 and Table II).

Comment. The influence of the daily intake of Cl^{35} on biologic decay rates of Cl^{36} has been demonstrated previously.[2,3] When the subject is in a steady state, the $C_{1/2}$ may be estimated from the mass of stable element in the body and the dietary intake. In these experiments the daily intake varied from 2.13 to 3.59 per cent of the average amount of stable element calculated to be in the body (Table III). The predicted $C_{1/2}$ values for Cl^{36} in the plasma for Subjects 1, 2, 3, and 4 were 20.0, 23.2, 19.3, and 32.6 days respectively, while the observed values were 14.9, 18.8, 22.8, and 28.0 days, respectively. These subjects, especially those with congestive heart failure, were not in a steady state since body weight changed.

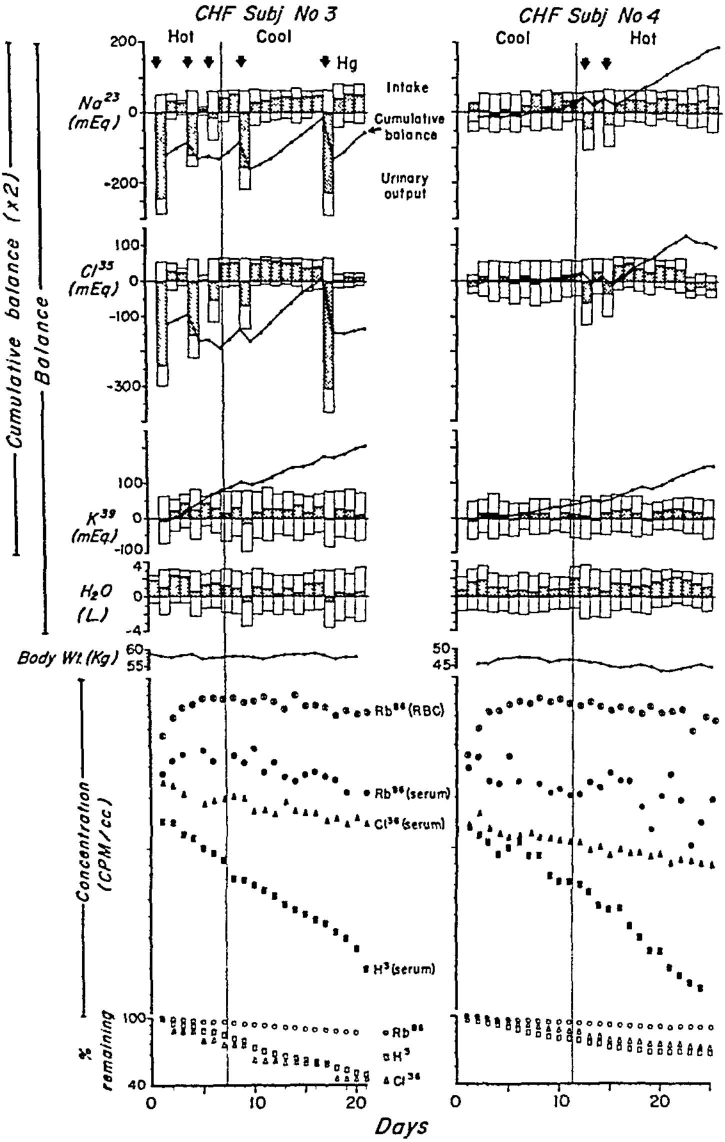

Fig. 3.—Biologic decay rates and metabolic balance data on 2 subjects with congestive heart failure. Manner of presentation is the same as in Fig. 2.

The $C_{½}$ values of Cl^{36} for the control subjects differed slightly from those with congestive heart failure. Since the daily intake of chloride was more than adequate to meet the demands of the hot environment, there were no significant differences in $C_{½}$ or $U_{½}$ values in the two environments. The effect of mercurial diuretics on $U_{1/2}$ is illustrated in Table II. Subject 3, for example, excreted most of the chloride during mercurial diuresis and thus had a $U_{1/2}$ value of 17.6 days when the entire study was considered and 96.7 days when the days of mercurial diuresis were excluded.

TABLE III. RELATIONSHIP OF INTAKE TO CALCULATED MASS OF WATER, CHLORIDE AND POTASSIUM

	WATER							
	S_o H^3 (0 TIME)		S_t H^3 (22 HOURS)		CALCULATED BODY WATER*		INTAKE	
SUBJECT	L	PER CENT B.W.	L	PER CENT B.W.	L	PER CENT B.W.	L	PER CENT 22 HR S_t
1	27.4	60.[illegible]	31.8	77.2	22.6	54.8	3.43	10.8
2	28.2	40.[illegible]	28.0	40.0	37.0	52.8	3.41	12.2
3	27.1	44.9	31.2	51.7	29.6	49.0	2.93	9.4
4	35.6	77.6	37.3	81.6	27.6	60.1	2.92	7.8
5	21.3		22.1					
Mean	27.9	57.2	30.1	62.6	29.2	54.1	3.2	10.1

	CHLORIDE				POTASSIUM			
	M_t, Cl^{36}		INTAKE		M_t, Rb^{86}		INTAKE	
SUBJECT	M_t (GM.)	PER CENT B.W.	GM.	PER CENT M_t	M_t (GM.)	PER CENT B.W.	GM.	PER CENT M_t
1	56.0† 72.6	0.152	1.94	2.67	144.0	0.344	2.48	1.72
2	63.6	0.093	1.90	2.99	117.0	0.172	2.63	2.25
3	53.0	0.091	1.90	2.59	104.4	0.179	2.92	2.80
4	88.8	0.193	1.89	2.13	143.9	0.313	2.17	1.51
5	51.5							
Mean	62.6	0.132	1.91	2.85	127.3	0.252	2.55	2.07

*Volume of body water calculated by the following formula: $M_o = \frac{I \times C_{1/2}}{0.693}$

†Average of first 4 days.

Radiorubidium. The $C_{1/2}$ and $U_{1/2}$ values for Rb^{86} show no effect of environment nor is there any obvious relation of these parameters of Rb^{86} to those of Cl^{36} (Table II). The concentration of Rb^{86} in the serum of all subjects varied considerably. The ratios of concentration of Rb^{86} in the erythrocytes to that in the serum are shown in Fig. 4.

The concentration time course curve of Rb^{86} in the erythrocytes revealed an increase in concentration of Rb^{86} for the first 8 to 11 days of observation. The $C_{1/2}$ value for the erythrocyte compartment was therefore expressed in two ways (Table II): (1) the $C_{1/2}$ value for the descending portion of the concentration time course after the peak concentration had been reached and (2) the $C_{1/2}$ value for erythrocyte compartment in which the ascending portion of the curve was included neglecting equilibrium of distribution. The value by the first method was essentially one half as long as that by the second method.

Comment. By definition of $C_{1/2}$ a state of equilibrium of distribution must first exist before this parameter can be measured. This state of equilibration probably never exists completely for an isotope of potassium or Rb^{86}. The error introduced by assuming that equilibrium of distribution was established is probably minimal between the fifth and twelfth days after injection[7, 8, 11]; however, the magnitude of the error is unknown.

Since the $C_{1/2}$ value for Rb^{86} based upon the entire concentration-time course curve in the erythrocytes was approximately twice that based upon the descending portion alone, the rate of exchange of Rb^{86} between erythrocytes and plasma must have remained essentially constant throughout the entire observation. Furthermore, when considering exposure of a specific compartment to the radiations of a radioactive element, the $C_{1/2}$ value derived from the entire concentration-time course curve of that compartment must be utilized.

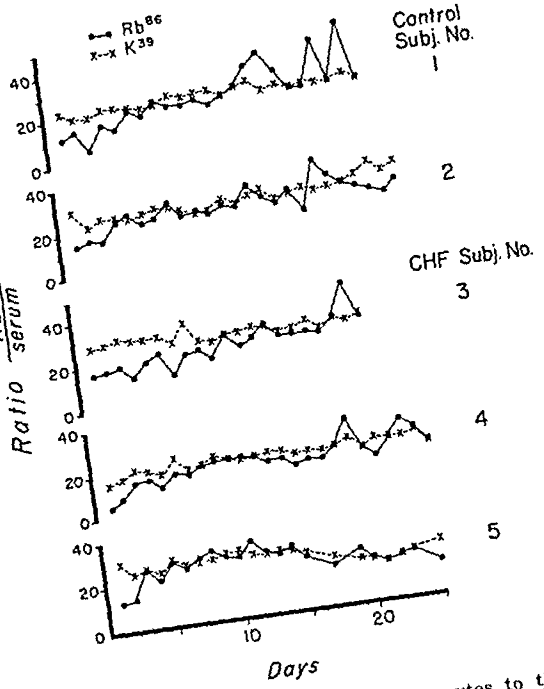

Fig. 1.—Time course of the ratios of K^{39} in the erythrocytes to that in the serum, and the ratios of Rb^{86} in the erythrocytes to that in the serum in each of the 5 patients. In only one subject (C.H.F. Subject 3) was there a prolonged lag in the time required for the ratio of Rb^{86} to approach the value of the ratio of K^{39}.

The concentration of Rb^{86} in plasma depends principally upon the rates with which various tissues take up and release Rb^{86}. Since the rate of decline in concentrations of Rb^{86} in the plasma was quite similar to that for the descending portion of the concentration-time course curve in the erythrocytes, it is apparent that the concentration in the plasma is dependent on release of Rb^{86} from organs. Furthermore, since only about 15 to 24 per cent of the injected Rb^{86} was recovered in the urine and the $C_{1/2}$ of plasma and erythrocyte compartments were similar and the ratios of Rb^{86} and K^{39} in the erythrocyte and that in the plasma were similar (Fig. 4), it is probable that the erythrocyte compartment reflects the kaliokinetic phenomena of the large cellular compartment of the body. The metabolic processes, which are concerned with exchange of Rb^{86} and potassium between the plasma and cells, were not influenced by the climatic conditions of this study.

The influence of intake of the stable element K^{39}, on the biologic decay rate of Rb^{86} is shown in Tables II and III Since the ingested K^{39} does not equilibrate quickly with the K and Rb of the entire body before excretion, the effect of varying intake of K^{39} on biologic decay rates would not be as predictable as for a substance which equilibrates more quickly such as chloride or water Nevertheless increase in intake of potassium reduced the $C_{½}$ values of Rb^{86} [8] In the subjects whose average daily intake of potassium was a greater percentage of the calculated potassium mass in the body a shorter time would have been required to recover one half of the injected Rb^{86} in the urine (Tables II and III) The administration of mercurial diuretics had little effect on urinary excretion of Rb^{86}

Tritium The biologic decay rates of tritium are shown in Figs 1 to 3 and in Table II There were no significant differences in $C_{½}$ values among 4 of the 5 subjects studied, however, one control subject had a small $C_{½}$ value There was no consistent effect of environmental temperature on these values The decay in concentration of tritium was remarkably constant throughout the observations The time course of the per cent of the tracer remaining showed a decrease in slope toward the end of the experiments (Figs 2 and 3), a behavior expected because of unmeasured extraurinary losses of tritium

Comment The values of $U_{½}$ were larger than those for $C_{½}$ because of extraurinary losses of water and tritium Therefore, the $C_{½}$ value was a better index of water turnover than $U_{½}$ All subjects were allowed water as desired *Although no purposeful water loading was attempted in these studies* Control Subject 1 had a very large daily intake of water and maintained a water diuresis throughout the study The smallest $C_{½}$ value was obtained in this subject The effect of intake of the nontracer water on biologic decay rates of H^3 would be expected as in the case of any isotope with which the ingested stable substance reaches a state of equilibrium of distribution in the body [6, 37]

The progressive decrease in the slope of the time course of the per cent of H^3 remaining in the body (not recovered in urine) could be due to fixation of H^3 in stable positions in the tissues The amount of tritium which would be expected to be bound in such stable chemical states would be very small during the course of the present studies, and would probably be within the small error of radioassay of H^3 Observations on animals[10, 17, 30, 33, 37, 38] have demonstrated the capacity of H^3 to be bound in stable positions and to lag thereafter in exchange with the hydrogen of plasma water If such stable binding involved a significant percentage of the injected tritium, one would expect the latter part of the concentration time course curve to show a slower rate of decline than the first part of the same curve since the percentage of H^3 remaining in the body which is chemically bound would increase progressively with time In these studies there were no apparent changes in the rate of decline of the concentration time course curves in plasma (Figs 1, 2 and 3) Although tritium traces hydrogen in the body with some "isotope effects," since the great mass of readily exchangeable hydrogen in the body is in the body water, the concentration time course curves and biologic decay rates in these studies reflected turnover of body water

The effect of environmental heat on the rate of loss of water from the body as sensible and insensible water loss is well known. Figs. 1 to 3 and Table II fail to show any detectable differences between the effects of the hot environment and the air-conditioned ward on the biologic decay rates of H^3. This does not mean that heat is unimportant in water loss from the body and in water turnover. Subjects 1 to 4 ingested approximately the same amounts of water each day in both hot and cool environments. When in the hot environment the decrease in volume of urine was offset by an increase in nonurinary loss of water so that water turnover, with approximately equal daily intake in both environments, would be expected to be the same. The $C_{½}$ values in these observations were between the extremes reported by Pinson[37] which reflects differences in water intake. As noted previously,[6, 38] the biologic half-life of an isotope which is freely exchanging in a compartment can be shortened by increasing the intake of the nontracer substance in that compartment.

Space and Mass.—Because of the long physical half-life of Rb^{86} and Cl^{36}, it was possible to calculate the time course of space and mass of chloride and potassium, assuming Rb^{86} traces K^{39} adequately. Calculations of the time course of tritium space were not justified because of the failure to measure nonurinary losses of H^3. The water space measured by tritium dilution was calculated for the time of injection and for 20 to 24 hours later. In Subject 5, incomplete collections made calculations of the time course of space and mass inaccurate.

The calculated electrolyte space and mass of the traced substance were defined as follows[39]:

S_0 = calculated tracer space or volume expressed in sampled compartment volume equivalents at t_0. This may be expressed in serum volume equivalents, erythrocyte volume equivalents, etc.

S_t = calculated tracer space at any time t.

M_0 = total calculated exchanging nontracer mass at t_0.

M_t = total calculated exchanging nontracer mass at any time t.

These values were calculated from the following equations:

$$S_0 = \frac{\text{amount of isotope injected}}{\text{concentration of isotope in sampled compartment at } t_0}.$$

$$S_t = \frac{\text{amount of isotope remaining in body at time t}}{\text{concentration of isotope in sampled compartment at time t}}.$$

M_0 = calculated space (S_0) × concentration of nontracer in sampled compartment at t_0.

M_t = calculated space (S_t) × concentration of nontracer in sampled compartment at time t.

The results are summarized in Table III and in Figs. 5 and 6. In Table III, the calculated space and, therefore, calculated mass of body water was also obtained from the following formula: $M_0 = \frac{I \times C_{½}}{0.693}$ where I is the daily intake of water.

The values for chloride mass and intake shown in Table III are averages over 20 to 25 consecutive days. Since Subject 2 was in a heavy body and leg cast, an accurate daily body weight was not available. Her weight was estimated to remain at 150 pounds.

The calculated values of potassium mass shown in Table III are averages of a number of daily M_t values obtained after the concentration time course of Rb^{86} in erythrocytes had achieved its maximal value. The days of the study included in these averages were as follows: Subject 1, ninth to fifteenth day; Subject 2, ninth to fifteenth day; Subject 3, thirteenth to twenty-sixth day; and Subject 4, eleventh to twentieth day.

Tritium: The tritium space at t_0 and at 22 hours calculated by dilution of tritium and the average mass of body water calculated from the average intake of water and C_{t_0} of H^3 varied as much as 20 per cent in the same subject (Table III). No one method of calculation gave consistently higher or lower values. The calculated body water varied from 54.8 to 77.2 per cent of body weight for Subject 1, 40.0 to 52.8 for Subject 2, 44.9 to 51.7 for Subject 3, and 60.1 to 81.6 per cent body weight for Subject 4.

Comment: The relatively large variations in calculated body water based on tritium dilution have been encountered by others.[12, 28, 37] The same variability was found with antipyrine and deuterium.[24, 40-43] The validity of the dilution techniques has been checked by desiccation techniques and determination of specific gravity of the body.[44] The reported wide variations are unreasonable. There is less water in fat than in other tissues, but it is difficult to explain the variations of body water from 40 to 80 per cent body weight on the basis of fat content alone. The average values for the 3 methods of calculation on the 4 subjects in this series were 57.2, 62 6, and 54.1 per cent, respectively, of body weight. The mechanism for the large day-to-day variations in a single individual is likewise difficult to explain Subject 2 was rather obese which may explain the relatively low value for body water but Subject 3 was quite thin and had edema and a considerable amount of ascitic fluid yet the calculated volume of body water was small. These variations indicate the difficulties associated with the tracer method of calculating space and mass of a substance in the body of man. There are very little data on reproducibility of body water measurements with tritium.[37] The extreme variability of calculated body water which has been reported may in part be related to the use of a single measurement of the tritium content of serum at the end of several hours after the injection of the tracer By extrapolating concentration time course curve based on numerous observations obtained over 1 to 2 days back to time of injection, the variability associated with a single determination should be avoided. A longer period of study would be satisfactory only if extrarenal losses of water were measured.

Chloride: The time courses of space (S_t) and mass (M_t) of chloride are shown in Figs. 5 and 6. The average daily mass expressed as per cent body weight, intake of Cl^{35} in both grams and as a per cent of average

M_t is shown in Table III. The time courses of calculated chloride space and mass showed a few variations in the first 3 to 4 days but thereafter both remained fairly constant. In Control Subject 1, there was a progressive rise in both calculated space and mass. The mass increased from an initial value of 52.9 Gm. at 24 hours to 91.7 Gm. at the end of 22 days reflecting extrarenal losses of Cl^{36} and Cl^{35} as well as inaccuracies of such measurements.

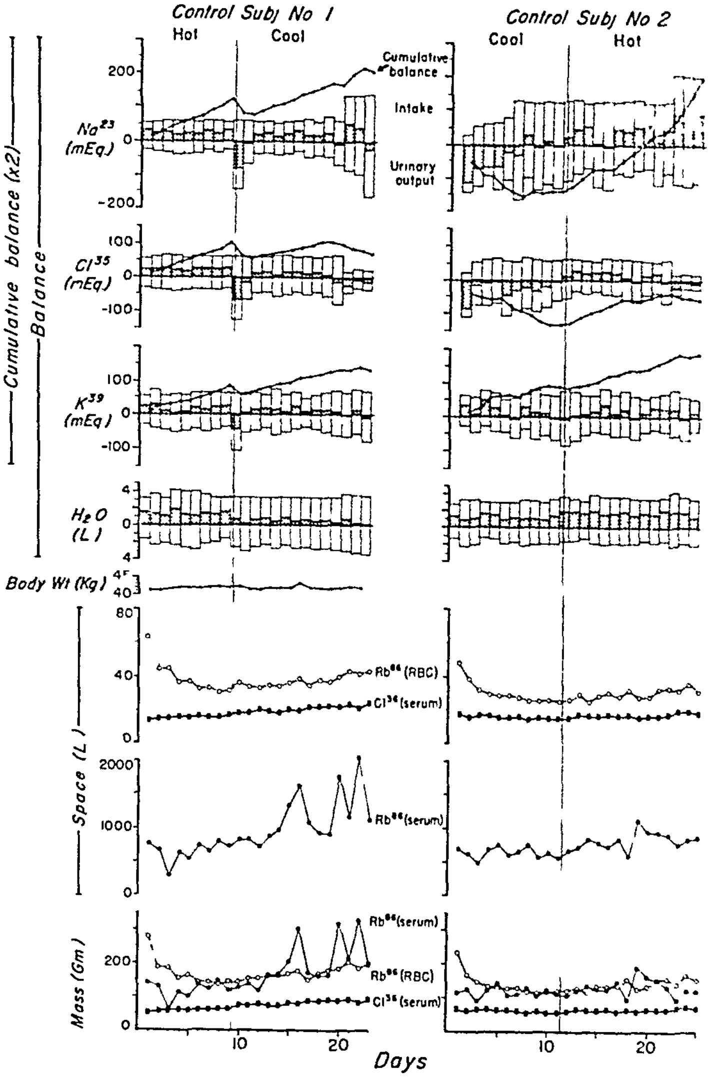

Fig. 5.—Time course of calculated space and mass of K^{39} and Cl^{35} in 2 control subjects. The data on electrolyte and water balance are presented as in previous figures. The time course of space of Rb^{86} (and therefore K^{39}) was calculated from the concentration of Rb^{86} in both the erythrocytes and serum. The calculated mass of K^{39} was also calculated from the Rb^{86} content of both compartments. The calculation of both space and mass of K^{39} is based on the assumption that Rb^{86} traces K^{39} adequately to justify such calculations. Both space and mass of Cl^{35} were calculated from the content of Cl^{36} in the serum.

There was no apparent effect of environmental temperature on the time course of S_t and M_t of chloride. The daily positive balance of Cl^{35} was greater in the hot environment which was most probably due to unmeasured extraurinary losses of Cl^{35} in sweat.

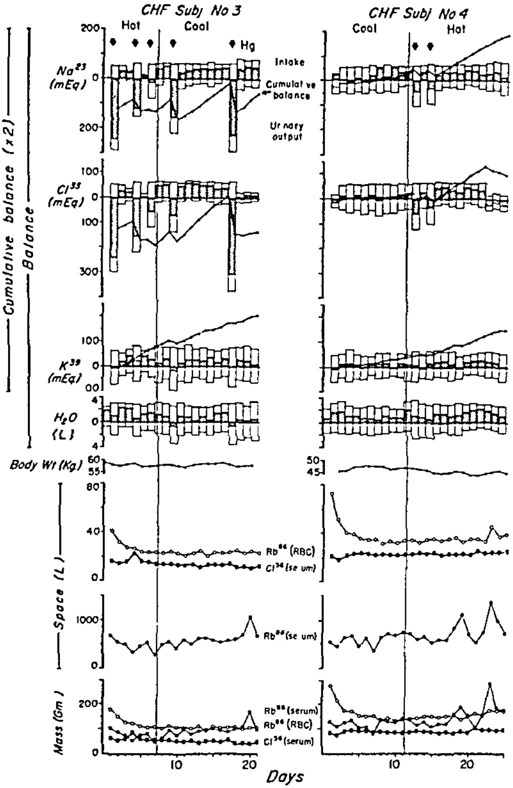

Fig 6.—Time course of calculated space and mass of K^{39} and Cl^{35} in 2 subjects with congestive heart failure. The manner of presentation is the same as in Fig 5.

Mercurial diuresis resulted in variations in calculated space and mass (Fig 6, Subject 3). The effects of administration of $NaHCO_3$ during the last 3 days of the study are shown in Figs 5 and 6.

Comment: In general, calculated space and mass of chloride bore a direct relationship to space and mass of body water in the control subjects and those with heart failure (Table III). The average chloride space as a per cent of body weight was 46.1 for Subject 1 (37.8 for the first 5 days), 23.5 for Subject 2, 24.5 for Subject 3, and 50.4 for Subject 4.

Radiorubidium: The time course of calculated space (S_t) and mass (M_t) of potassium for both erythrocyte and plasma compartments are shown in Figs. 5 and 6.

The time course curves of calculated space and mass based on the erythrocyte compartment showed a decline for the first 7 to 10 days followed by a fairly constant value. The time course of S_t and M_t based on the plasma compartment shows rather wide daily fluctuations, although in general these curves were parallel to those based on the erythrocyte compartment. There was no apparent effect of the hot or cool environment on these parameters in any of these subjects.

In all subjects there was an expected progressive increase in the cumulative positive balance of K^{39} due to extrarenal losses. The calculated space and mass of K^{39} varied directly with that for water and chloride.

Comment: These studies add further to the data which show Rb^{86} to trace K^{39} fairly well in intact man.[2, 8, 45] There is no way to determine precisely when equilibrium of distribution of Rb^{86} has occurred. However, by studying the major compartments of K^{39}, such as the intracellular or erythrocyte compartments, a better estimate of the mass and kinetics of the traced element is obtained. The plasma and extracellular compartments are relatively small and, therefore, likely to vary considerably in concentration of Rb^{86}. The general parallelism between the time course of S_t and M_t based upon Rb^{86} in both erythrocyte and plasma compartments indicates the dependence of the plasma compartment on release of Rb^{86} from the major (intracellular) compartment. In only one patient (Subject 3) was there a long lag in the time required for the time course curve of M_t based on plasma compartment to reach the level of the curve based on erythrocyte compartment (Fig. 6).

The cellular metabolic phenomena which are related to the incorporation of Rb^{86} and potassium into the cellular compartment were not altered by environmental temperature during these studies. The rates of exchange of Rb^{86} between the cellular compartment and the plasma continued at the same rate throughout the experiment.

DISCUSSION

For the first time simultaneous studies of the kinetics of tritium, rubidium and chloride as well as aspects of the metabolism of sodium, chloride, potassium, and water have been made in man under comfortable and hot and humid environmental conditions to which both healthy and sick people of many areas of the world may be subjected. These studies failed to reveal any definite differences attributable to the environmental temperature in either the control subjects or in the patients with chronic congestive heart failure. In spite of this, however, except for one patient (Subject 4) they were all impressed by the benefits of the comfortable environment upon their well being and by the oppressiveness of the hot and humid environment. This was particularly true of the patient in the heavy body and leg cast. The attendants, physicians, and investigators were likewise impressed by these differences. More clinical data will be published elsewhere concerning these reactions. That over all differences existed and the fact that the patients perspired less, were more relaxed, and clinically better was evident in spite of the experimental data. The psychologic reactions of the patients to these environments are especially in need of study.

However, in spite of these clinical differences, these detailed studies of metabolism of electrolytes and water failed to reveal any definite differences in equilibrium of distribution, biologic decay rates, or calculated space and mass of electrolytes and water. This is not surprising in view of the precipitous decline in the urinary excretion of sodium and chloride even though there is measurable change in the Na or Cl concentration in the plasma when *a subject is placed on a low dietary intake of salt. In other words* the regulation of any single physiologic phenomenon may be so sensitive that important differences in physiologic reactions can exist with immeasurable differences in the regulating phenomena responsible for the changes. To know that this is true for the metabolic and kinetic biologic phenomena of water and electrolytes is important.

The greatest differences, although not too large in magnitude, were noted in certain aspects of renal excretory function which will be described elsewhere.[47] It was rather interesting that the group of subjects under study drank large quantities of water each day. The intake far exceeded the needs for the comfortable environment and was more appropriate for the needs for the hot and humid environment. It is quite possible that, during the process of developing acclimatization as the New Orleans summer developed, these patients drank more and more water which became a habit to be maintained for a time regardless of need. Therefore, when they entered the comfortable environment they continued to drink large quantities of water by force of habit rather than for physiologic requirements. Furthermore, the large intake of water and the resultant diuresis inhibited any manifestations of the environmental effects of excretion of tritium and its biologic half life. Since output of H_2O was determined by a very high intake, mechanisms for conservation of H_2O did not have to develop. Thus, total excretion of H^3, regardless of the proportion in sweat and in urine, was high and fairly constant (Figs. 1 to 3). Had H_2O intake been limited to minimal urinary needs in the neutral or comfortable environment, this intake would have been inadequate in the hot environment when H_2O was deviated into sweat for thermal regulation.

The experiments demonstrate the possibility of employing several radioactive isotopes simultaneously for physiologic and pharmacologic studies over prolonged periods of time in intact man. The tracers were readily separated with accurate counting. The findings with the simultaneous studies of several tracers were the same as obtained previously in studies of individual isotopes and, therefore, increase the series for control subjects and for patients with chronic congestive heart failure. The various illustrations reveal the temporal qualitative and quantitative relationships of the 3 tracers used and interrelationships of physiologic adjustments involving water, chloride, and potassium.

SUMMARY AND CONCLUSIONS

Simultaneous studies of Cl^{36}, Rb^{86}, and H under controlled metabolic conditions were made in both control subjects and in patients with congestive heart failure. The effect of a comfortable air-conditioned ward and of a hot and humid environment on kinetics and metabolism of electrolytes and water in both groups of subjects was studied. Even though there was little measurable effect of environmental temperature on the parameters observed in this study, there were rather distinct differences in the effect of the environment from a clinical standpoint. Possible reasons for the discrepancies between physiologic and clinical data have been discussed.

These experiments show the feasibility of long-term metabolic studies using a combination of isotopes to observe extracellular and intracellular electrolyte metabolism simultaneously with studies of turnover of body water. Additional data on the biologic decay rates of Rb^{86} and Cl^{36} have been presented along with data on the measurement of body water with tritium.

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STUDIES OF THE INFLUENCE OF COMFORTABLE AND HOT AND HUMID ENVIRONMENTS UPON THE RATE OF EXCRETION OF H^3, H_2O, Cl^{36}, Cl^{35}, Na^{23}, Rb^{86}, AND K^{39} IN CONTROL SUBJECTS AND PATIENTS WITH CONGESTIVE HEART FAILURE

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STUDIES were initiated to investigate the influence of a hot and humid hospital environment of New Orleans during the summer upon certain phases of electrolyte and water metabolism in subjects with and without chronic congestive heart failure. Other aspects of these experiments have been described in a preceding paper.[1] The hot and humid environment produced definite changes in the urinary excretion of water and electrolytes associated with an increase in sweating in most of the subjects. Contrastingly, the comfortable air conditioned ward revealed the influence of air conditioning upon renal and sweat gland function.

METHODS AND MATERIALS

Nine women were studied under controlled metabolic conditions in the two wards of Charity Hospital (Table I). One ward was air conditioned (mean temperature 74.4° F., range 80.2° maximum to 70.1° minimum) with a mean relative humidity of 73.4 per cent (range 90 to 60 per cent), while the environment of the other ward was hot and humid—the weather existing in New Orleans in July and August, 1956 (mean temperature *83.4° F., range 94.0° maximum to 69.0° minimum), with a mean relative humidity of 80.0* per cent (range 98 to 47 per cent). The maximum daily temperature was 90.0° F. or greater on 21 of the 26 days of this study. The average daily temperature recorded twice daily on the ward was 86.8° F. with a relative humidity of 62.0 per cent. The subjects in this study spent approximately equal time in both environments. One half of the patients were in the comfortable environment first and, after approximately 2 weeks, were transferred to the other environment. At the onset of these studies 5 subjects received intravenously Cl^{36}, Rb^{86}, and H^3OH simultaneously.

Dietary intake of sodium, chloride, potassium, and water was recorded and the urinary excretion of these substances was measured. No measurements were made of the sweat or stools.

Blood was collected under oil each morning, and the plasma and whole blood were studied separately. The following measurements were made on the plasma: sodium and potassium by flame photometry, chloride by the method of Schales and Schales,[2] osmolarity by the Fiske osmometer, creatinine by the method of Haugen[3], Rb^{86} and Cl^{36} by

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end window Geiger Müller counters,[1] and H^3 with a liquid scintillation counter.* After laking by freezing and thawing of an aliquot of whole blood, the sodium, potassium, Rb^{86}, Cl^{36}, and H^3 were measured.

The 24 hour urine specimens, collected from 8:00 A.M. to 8:00 A.M. with toluene as the preservative, were studied by the above methods for sodium, chloride, potassium, Rb^{86}, Cl^{36}, H^3, osmolarity, and creatinine. Urinary volume, specific gravity, and pH were also determined.

Separate radioassay of Rb^{86} and Cl^{36} was accomplished by counting soon after collection of the samples and recounting the preparations 200 days later after Rb^{86} had undergone physical decay. Radioassay of tritium was done on a water distillate of the biologic fluids which were distilled at low temperatures so that Cl^{36} did not enter the distillate.

Dietary intake of sodium, potassium, chloride, and calories was kept relatively constant, but water was allowed as desired. The subjects received various drugs and therapeutic measures as indicated by the clinical state (Table I).

TABLE I. CLINICAL DATA ON THE NINE SUBJECTS STUDIED

SUBJECT NO.	AGE (YR.)	DIAGNOSIS	DOSE OF ISOTOPES Cl^{36} (MC.)	Rb^{86} (MC.)	H^3 (MC.)	TREATMENT
1	46	*Control; respiratory* infection, convalescent	0.026	0.631	3.749	*None*
2	50	Control; tuberculous arthritis	0.027	0.663	4.104	Streptomycin, sodium para-aminosalicylate, body and leg cast
3	52	R.H.D., C.H.F.	0.020	0.481	2.993	Digitalis, mercurial diuretics
4	52	A.S.H.D., C.H.F., H.H.D.	0.027	0.662	3.780	Digitalis, mercurial diuretics
5*	65	A.S.H.D., empyema, C.H.F., pneumothorax	0.026	0.642	3.852	Digitalis, thoracentesis, penicillin
6	23	Control; sarcoidosis				Prednisone, 30 mg. daily
7	32	Control; lung abscess, convalescent				Penicillin, Achromycin
8	22	R.H.D., C.H.F.				Digitalis, prednisone, 30 mg. daily
9	56	A.S.H.D., mild angina, C.H.F.				Digitalis, mercurial diuretics

Key to abbreviations: R.H.D. = rheumatic heart disease; C.H.F. = congestive heart disease; A.S.H.D. = arteriosclerotic heart disease; H.H.D. = hypertensive heart disease.

*Incomplete urine collections.

RESULTS

The results are summarized in Figs. 1 to 8 and in Tables II, III, and IV. The data are presented as follows: clearance rates; daily excretion of chloride and potassium as a per cent of calculated mass; and osmolar and free water excretion, glomerular filtration, and renal tubular reabsorption.

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*The authors wish to express their appreciation to Drs. Leon Jacobsen and George Okita of the University of Chicago School of Medicine for the use of their laboratory facilities for counting the tritium samples.

Urinary Clearance Rates—Average daily clearance rates were determined for Cl^{36}, Cl^{35}, Na^{23}, Rb^{86}, K^{39}, and H^3 in 4 subjects (Figs 1 and 2) and for Cl^{35}, Na^{23}, and K^{39} in 4 additional subjects (Figs 3 and 4)

Rb^{86} and K^{39} clearances The qualitative and quantitative similarities of the clearance rates of Rb^{86} and K^{39} are evident Variations exist in rate of clearance of potassium without an equal quantitative change for Rb^{86}, but qualitative differences were few (12 points of qualitative directional differences in 4 subjects) (Figs 1 and 2) That potassium was excreted preferentially over Rb^{86} is apparent from these graphs, but Rb^{86} traced K^{39} reasonably well the correlation coefficient being +0 816 This is approximately the same relationship for excretory rates previously described[5]

The average daily rates of clearance of potassium varied widely among the subjects and in the same subject from time to time, the minimal rate being about 4 ml of plasma cleared per minute and the maximal rate about 18 ml per minute The magnitude of variations within each individual subject was almost as great as the variations among the subjects There was no apparent effect of the clinical state of congestive heart failure or of the two environmental conditions on the average rate of clearance of K^{39} or Rb^{86} Subject 1 had a very high rate of clearance on the first day in the cool environment The intake of potassium was higher *in Subject 8 who was given added KCl along with prednisone* The average rates of clearance of K^{39} in this subject and in Subject 6, who likewise received prednisone, were greater than in the other subjects (Figs 1 to 4)

Administration of a mercurial diuretic had a variable effect on the rates of clearance of K^{39} and Rb^{86} (Figs 2 and 4)

Comment The quantitative differences in rates of clearance of K^{39} and Rb^{86} reflect a difference between these two isotopes which was greater for the kidney than for any other organ studied to date[3, 4] The reason for this difference in excretory rates is not apparent but may be related to differences in atomic masses in spite of about equal sizes for the hydrated ions

The absolute values of clearance of K^{39} among the various subjects as expected, reflected the variations in daily intake of potassium, which varied from 52 to 79 mEq per day in 7 subjects and was as high as 118 mEq per day in the subject who received added KCl However, the day to day intake of potassium varied relatively little for any given subject Such small daily variations in intake in any subject were insufficient to explain the marked differences in daily rates of clearance of potassium Furthermore, the change in intake and rate of clearance were not always directionally related Interestingly, the clearance rates were not influenced by the environments employed The effects of prednisone on excretion and balance of potassium are shown by the negative balance in Subject 6 Since nonurinary losses of K^{39} were not measured, the exact deficit of potassium in this subject was not measurable The progressive increase in cumulative balance of potassium in all other subjects reflected, in large part, nonurinary loss

Tritium clearance: The rates of clearance of H^3 for 4 subjects are summarized in Figs. 1 and 2. In Subjects 1 and 3, the rates of clearance were slightly higher in the cool environment. The various "isotope effects" due to greater atomic mass and other physical phenomena which have been ascribed to H^3 as a tracer of H^1 were not reflected in the urinary clearance rates as indicated by the correlation coefficient of clearance of H^3 to water clearance of +0.969.

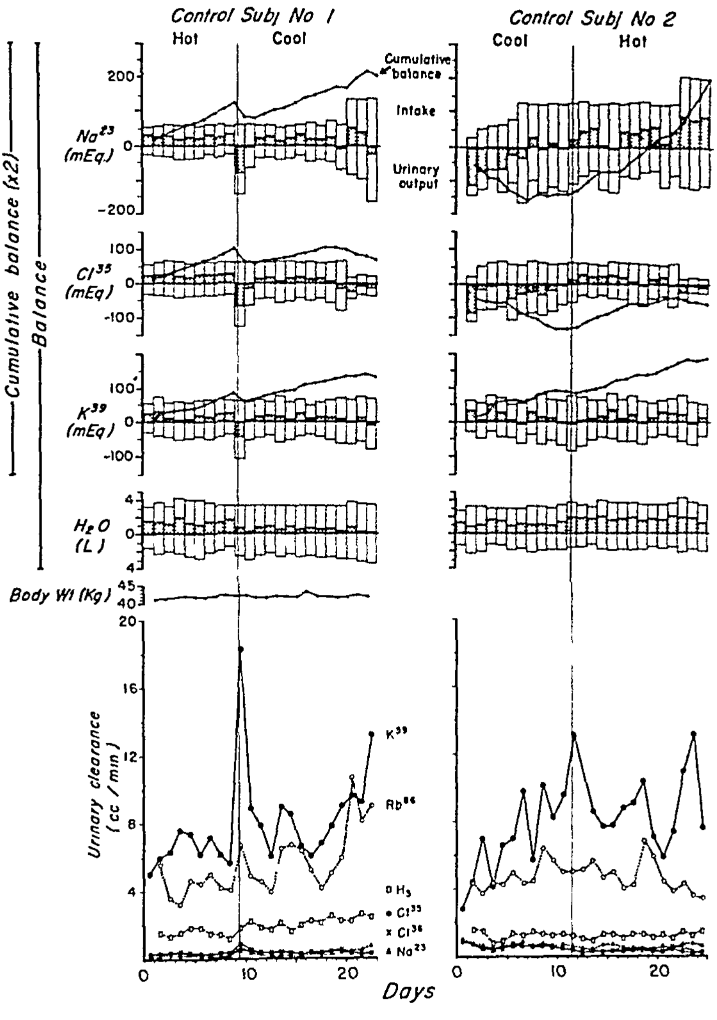

Fig. 1.—Time course of urinary clearance of K^{39}, Rb^{86}, H^3, Cl^{35}, Cl^{36}, and Na^{23} in 2 control subjects. In this and all subsequent figures the balance data is shown in the upper part of the figure. The ordinate values for cumulative balance are twice those shown for daily intake, urinary output, and balance. The values for average daily urinary clearances of the radioisotopes and nontracer elements are shown in the lower part of the figure. Body weights were not obtainable on Control Subject 2 since she was in a body and leg cast.

When mercurial diuretics and spontaneous factors increased the rates of clearance of Na^{23} and Cl^{35}, clearance of H^3 increased. The changes in these clearances varied concordantly yet they differed quantitatively. The clearance rates of H^3 varied from 0.5 ml. to 2.7 ml. per minute in Subject 3 as a result of mercurial diuresis. The other subject with congestive heart failure (Subject 4) had slightly lower rates of clearance of H^3 than the control subjects (Subjects 1 and 2).

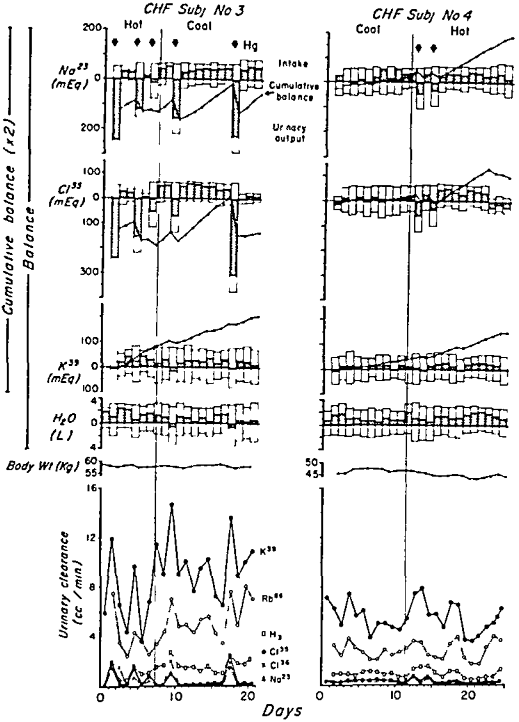

Fig 2.—Time course of urinary clearance of K^{39}, Rb^{86}, H^3, Cl^{35}, Cl^{36}, and Na^{23} in 2 subjects with congestive heart failure. See legend for Fig 1.

Comment: The rate of urinary clearance of H^3 varied directly with water intake and inversely with the rate of nonurinary water loss. Since these patients (Subjects 1 to 4) had little change in weight, the rate of clearance of H^3 reflected the water drinking habits of the individual. The correlation coefficient of +0.969 between the rates of clearance of H^3 and the rates of clearance of plasma water indicated the absence of skimming, isotope effects due to differences in mass of H^1 and H^3, and the excretion of significant amounts of H^3 in molecular combinations other than in water.

The relationship of rates of clearance of H^3 to that of Cl^{36}, Na^{23}, and K^{39} indicates that the subjects who were allowed water as desired drank sufficient

Table IV. The Influence of Heat and [illegible] Urinary Excretion of Solute

SUBJECT NO.	MOSM. FILTERED (MOSM./MIN.)			MOSM. EXCRETED (MOSM./MIN.)			MOSM. REABSORBED (MOSM./MIN.)			ENDOGENOUS CREATININE CLEARANCE (ML./MIN.)		
	ENTIRE STUDY	HOT	COLD	ENTIRE STUDY	HOT	COLD	ENTIRE STUDY	HOT	COLD	ENTIRE STUDY	HOT	COLD
1	21.93	21.19	22.40	0.295	0.266	0.313	21.63	20.93	22.09	79.10	77.18	80.34
2	19.47	17.81	21.58	0.338	0.343	0.331	19.13	17.47	21.25	67.51	64.42	78.58
3*	21.03	17.02	23.03	0.336	0.310	0.348	20.69	16.71	22.69	74.63	60.33	81.78
4*	16.13	16.69	15.43	0.299	0.304	0.293	15.83	16.38	15.13	59.04	61.34	56.13
6	31.49	30.37	32.37	0.523	0.554	0.506	30.96	29.81	31.86	113.46	109.53	116.54
7	24.97	24.86	25.11	0.260	0.223	0.299	24.70	24.56	24.88	92.11	91.60	92.75
8*	24.22	24.55	23.96	0.497	0.423	0.555	23.72	24.13	23.40	87.14	89.12	85.59
9*	31.10	31.47	30.78	0.436	0.410	0.460	30.66	31.06	30.32	109.18	110.31	108.19
Average	23.79	23.00	24.33	0.373	0.354	0.388	23.42	22.63	23.95	85.27	82.98	87.49

*Congestive heart failure.

result of mercurial diuresis The other subject with congestive heart failure (Subject 4) had slightly lower rates of clearance of H^3 than the control subjects (Subjects 1 and 2).

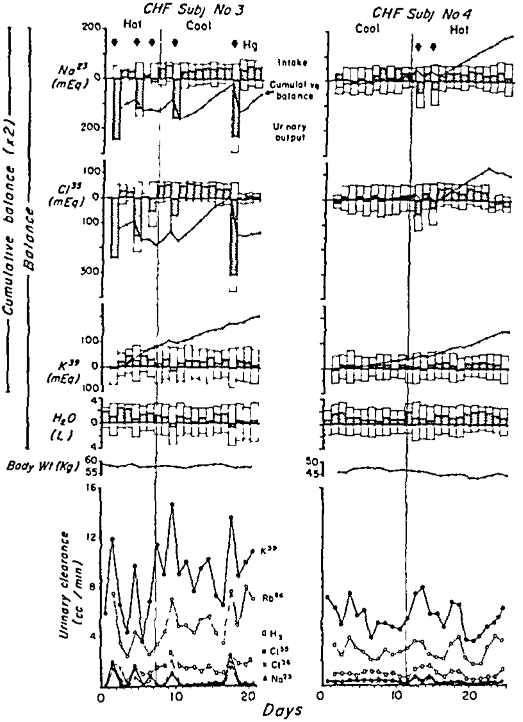

Fig 2—Time course of urinary clearance of K^{39}, Rb^{86} H^3, Cl^{35}, Cl^{36}, and Na^{23} in 2 subjects with congestive heart failure See legend for Fig 1

Comment: The rate of urinary clearance of H^3 varied directly with water intake and inversely with the rate of nonurinary water loss Since these patients (Subjects 1 to 4) had little change in weight, the rate of clearance of H^3 reflected the water drinking habits of the individual. The correlation coefficient of +0.969 between the rates of clearance of H^3 and the rates of clearance of plasma water indicated the absence of skimming, isotope effects due to differences in mass of H^1 and H^3, and the excretion of significant amounts of H^3 in molecular combinations other than in water.

The relationship of rates of clearance of H^3 to that of Cl^{36}, Na^{23}, and K^{39} indicates that the subjects who were allowed water as desired drank sufficient water to maintain approximately the same rate of free water excretion for both environments.

Cl^{36}, Cl^{35}, and Na^{23} clearances: The rates of clearance of Cl^{35} and of Cl^{36} had a correlation coefficient of +0.949 (Figs. 1 and 2). This value should be unity, but the low levels of counts of Cl^{36} in some specimens of urine increased the counting error. The time course of the relationship of the rates of clearance of Cl^{36} to Cl^{35} is shown for 4 subjects in Figs. 1 and 2.

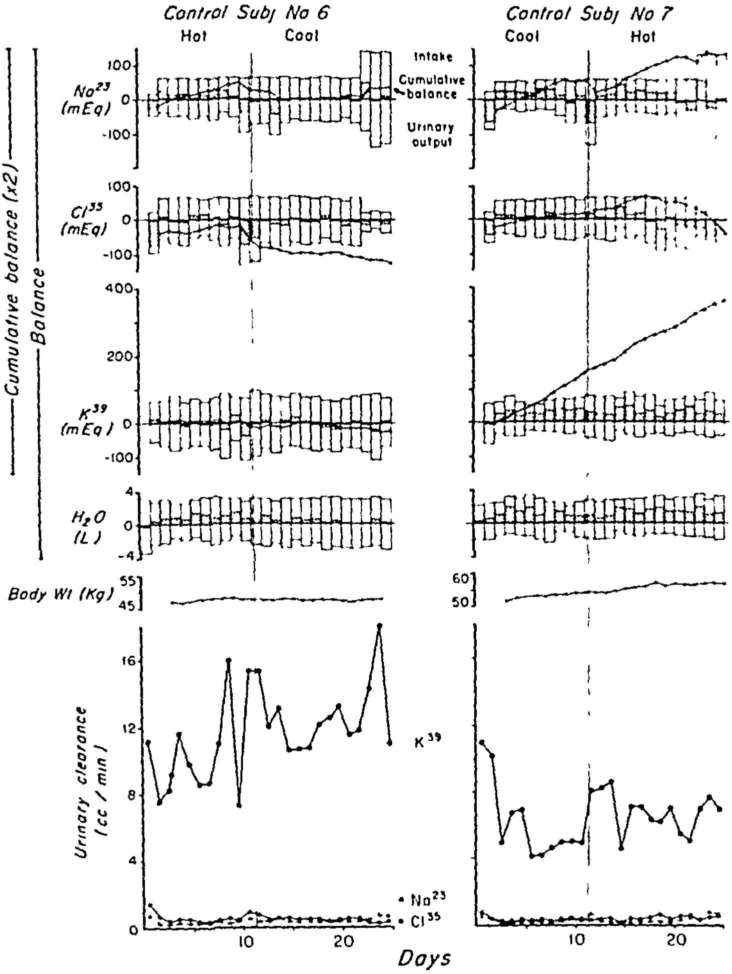

Fig. 3.—Time course of urinary clearance of K^{39}, Cl^{35}, and Na^{23} in 2 control subjects. See legend for Fig. 1.

The rates of clearance of Cl^{35} and Na^{23} were much lower than those for Rb^{86}, H^3, and K^{39} (Figs. 1 to 4 and Table II). The relationship of these rates of clearance to sodium and chloride balance are summarized in Figs. 1 to 4. The rate of clearance of sodium was approximately 75 per cent that of chloride in Subjects 6, 7, and 9; however, in Subject 2, the clearance of sodium was slightly greater than that for chloride and in Subject 8 the clearance of Cl was considerably greater than that for Na, these differences among subjects being related to intake of these electrolytes. There was no consistent effect of environment on these rates of clearance. The effects of mercurial diuresis on these subjects made the effect of environment difficult to evaluate.

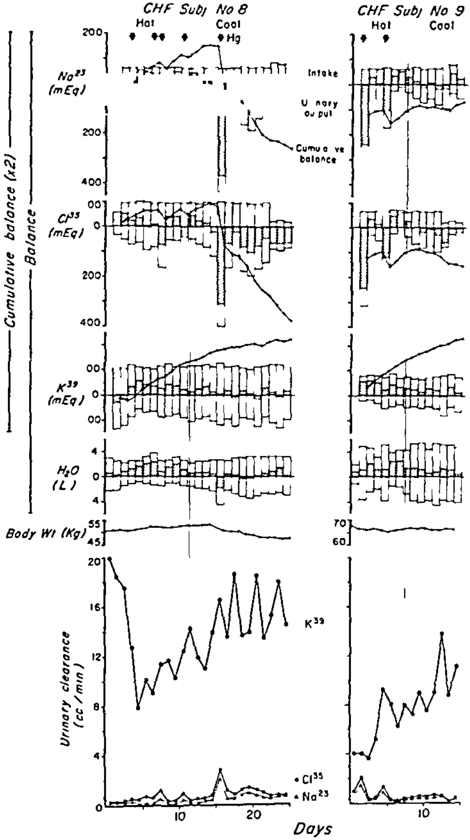

Fig. 4.—Time course of urinary clearance of K^{39}, Cl^{35} and Na^{23} in 2 subjects with congestive heart failure. See legend for Fig. 1.

Comment. The well known effects of dietary intake of sodium and chloride on the respective clearance rates are evident in the illustrations. In none of the subjects were there any significant changes in the concentration of chloride and sodium in the serum.

TABLE II. THE EFFECT OF ENVIRONMENT ON URINARY CLEARANCE RATES

SUBJECT NO.	PERIOD STUDIED	AVERAGE CLEARANCES (ML./MIN.) K^{39}	Rb^{86}	Cl^{36}	Cl^{35}	Na^{23}	H^3	AVERAGE DAILY URINE VOLUME (ML.)
1	Entire study	8.13	5.55	0.30	0.32	0.26	1.87	2,592
	Hot	6.55	4.32	0.23	0.26	0.16	1.46	2,249
	Cold	9.04	6.26	0.34	0.36	0.32	2.09	2,787
2	Entire study	8.42	4.73	0.36	0.37	0.55	1.24	1,790
	Hot	9.23	4.72	0.25	0.27	0.50	1.23	1,780
	Cold	7.27	4.76	0.52	0.52	0.61	1.27	1,800
3	Entire study	9.08	4.94	0.46	0.44	0.32	1.44	2,009
	Hot	7.15	3.97	0.75	0.73	0.44	0.98	1,391
	Cold	9.91	5.36	0.34	0.32	0.28	1.64	2,273
4	Entire study	5.53	2.86	0.29	0.29	0.22	0.92	1,595
	Hot	5.60	2.93	0.29	0.27	0.21	0.94	1,556
	Cold	5.42	2.75	0.29	0.31	0.24	0.90	1,651
6	Entire study	11.72			0.45	0.34		1,640
	Hot	10.45			0.47	0.26		1,348
	Cold	12.64			0.43	0.39		1,849
7	Entire study	6.42			0.38	0.28		1,591
	Hot	6.85			0.42	0.34		1,612
	Cold	5.83			0.34	0.21		1,561
8	Entire study	13.71			0.78	0.41		2,164
	Hot	12.16			0.54	0.18		1,364
	Cold	14.81			0.95	0.58		2,735
9	Entire study	7.94			0.54	0.38		3,355
	Hot	6.12			0.70	0.50		2,574
	Cold	9.30			0.42	0.30		3,940

Some of the effects of the environment observed for Control Subjects 1 and 2 might be expected on the basis of loss of sodium and chloride in sweat because of the hot and humid environment. The cardiovascular stress of temperature regulation has been indicated previously.[7] Among the 4 patients with congestive heart failure there was only one instance where the clinical state of failure was influenced by the environment if this is reflected in excretory rates. The spontaneous diuresis which developed in Subject 8 on entering the cool and comfortable environment may have been the result of relief from the cardiovascular burden of heat regulation. Like lessening the load of muscular exercise by bed rest, lessening the burden of body temperature regulation may have resulted in the diuresis.

Urinary Excretion of Cl^{35} and K^{39} as a Per Cent of Calculated Mass.—The mass of Cl^{35} and of K^{39} was calculated from tracer dilution in 4 patients and daily excretion was expressed as a per cent of the "calculated" masses (Table III). The excretion of chloride was expressed as a per cent of average daily M_t for the entire study whereas excretion of K^{39} was expressed as a per cent of the estimated K^{39} mass during the period of days when this value was constant.

Since there was a rise in calculated M_t for potassium toward the end of the experiment as a result of cellular lags in exchange of Rb^{86} with plasma the period of relatively constant M_t was selected for these calculations

TABLE III EFFECT OF ENVIRONMENT ON URINARY EXCRETION OF CHLORIDE AND POTASSIUM

SUBJECT NO	DAILY EXCRETION AS A PER CENT OF THE AVERAGE CALCULATED MASS					
	Cl^{35}			K^{39}*		
	ENTIRE STUDY	HOT	COLD	ENTIRE STUDY	HOT	COLD
1	2.35	1.79	2.73	1.40	1.17	1.55
2	3.36	2.31	4.72	1.67	1.81	1.50
3†	4.29	6.48	3.19	1.96	1.48	2.20
4†	1.78	1.67	1.93	1.17	1.15	1.20

*Calculated from data on Rb^{86}

†Congestive heart failure

Chloride The excretion of Cl^{35} as a per cent of calculated M_t for the 2 control subjects differed considerably (Table III) Subject 1 excreted about 2 per cent of her Cl^{35} mass per day with a peak rate noted on the day of transfer into the cool environment Subject 2 excreted more chloride during the exposure to the cool environment (average about 4 per cent) than while she was in the hot and humid one (average 2.5 per cent) Subject 3 with congestive heart failure excreted slightly less than 1 per cent of M_t on the days when she did not receive mercurial diuretics The profound effect of mercurials on excretion of chloride was demonstrated in this subject who excreted about ¼ of the chloride calculated to be in her body in one day of mercurial diuresis The other patient with congestive heart failure (Subject 4) excreted about 2 per cent of M_t daily while in the cool environment In the hot and humid environment there was a decrease in excretory rate on the days when she did not receive a mercurial diuretic

Comment During periods of zero balance of any electrolyte, the urinary excretion should represent the same per cent of calculated M_t as does intake provided there are no important extraurinary losses of these substances The dietary intake of chloride was 2.7, 3.0, 3.6, and 2.1 per cent of calculated M_t for Subjects 1 to 4, respectively

Potassium The time course of daily excretion of potassium expressed as a per cent of the average calculated M_t did not bear any directional relation with that of chloride except on the days of mercurial diuresis in Subjects 3 and 4 (Fig 2) and in Subject 1 on her first day in the cool environment The average per cent of M_t excreted per day was 1.2, 1.5, 1.9, and 1.1 for Subjects 1 to 4 respectively, the range being 0.6 to 3.4 per cent The average dietary intake of potassium was 1.7, 2.3, 2.8, and 1.5 per cent of average calculated M_t for Subjects 1 to 4, respectively There was no effect of change in the environment on the rates of excretion of potassium except in Subject 1 (Fig 1) who had a marked diuresis of K^{39} on being transferred from the hot and humid to the comfortable environment

TABLE IV. THE INFLUENCE OF ENVIRONMENT ON THE URINARY EXCRETION OF SOLUTE

SUBJECT NO.	MOSM. FILTERED (MOSM./MIN.)			MOSM. EXCRETED (MOSM./MIN.)			MOSM. REABSORBED (MOSM./MIN.)			ENDOGENOUS CREATININE CLEARANCE (ML./MIN.)		
	ENTIRE STUDY	HOT	COLD	ENTIRE STUDY	HOT	COLD	ENTIRE STUDY	HOT	COLD	ENTIRE STUDY	HOT	COLD
1	21.93	21.19	22.40	0.295	0.266	0.313	21.63	20.93	22.09	79.10	77.18	80.34
2	19.47	17.81	21.58	0.338	0.343	0.331	19.13	17.47	21.25	67.51	64.42	78.58
3*	21.03	17.02	23.03	0.336	0.310	0.348	20.69	16.71	22.69	74.63	60.33	81.78
4*	16.13	16.69	15.43	0.299	0.304	0.293	15.83	16.38	15.13	59.04	61.34	56.13
6	31.49	30.37	32.37	0.523	0.554	0.506	30.96	29.81	31.86	113.46	109.53	116.54
7	24.97	24.86	25.11	0.260	0.223	0.299	24.70	24.56	24.88	92.11	91.60	92.75
8*	24.22	24.55	23.96	0.497	0.423	0.555	23.72	24.13	23.40	87.14	89.12	85.59
9*	31.10	31.47	30.78	0.436	0.410	0.460	30.66	31.06	30.32	109.18	110.31	108.19
Average	23.79	23.00	24.33	0.373	0.354	0.388	23.42	22.63	23.95	85.27	82.98	87.49

*Congestive heart failure.

Osmolar Excretion, Glomerular Filtration, and Tubular Reabsorption.—The data of plasma and urine osmolarity, osmolar, free and total water clearance rates, milliosmolar glomerular filtration rates, and endogenous creatinine clearance rates are summarized in Figs. 5, 6, 7, and 8 and Table IV.

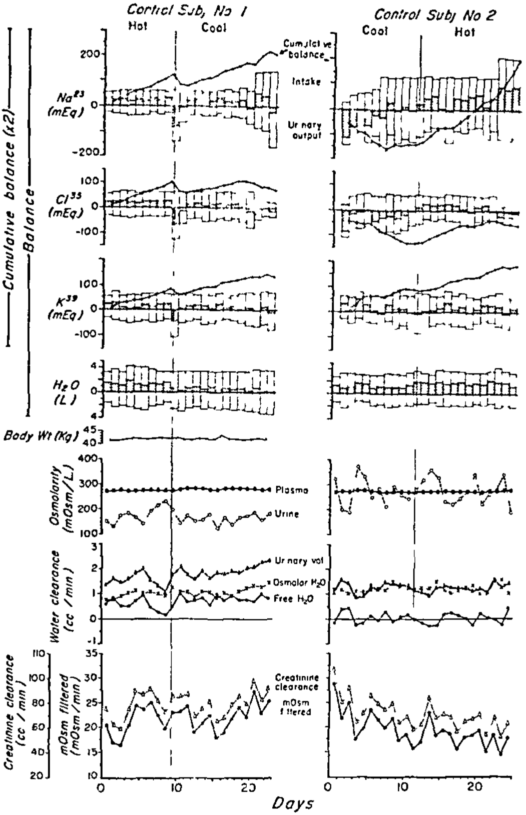

FIG 5.—Time course of urinary excretion of water in 2 control subjects The balance data is presented as in Fig 1 In addition, the osmolarity of plasma and urine, urinary volume, osmolar and free water clearance, and endogenous creatinine clearance and milliosmoles filtered are shown in the lower part of the charts

Plasma osmolarity changed very little throughout the entire period of observation. Urinary osmolarity, on the other hand, showed considerable variation among the subjects as well as in the same subject in the two environmental conditions. In Control Subjects 1 and 2 (Fig. 5) there was no difference in osmolarity of the urine in the two environments, while in the remaining 6 subjects the urine osmolarity was higher when they were in the hot and humid environment.

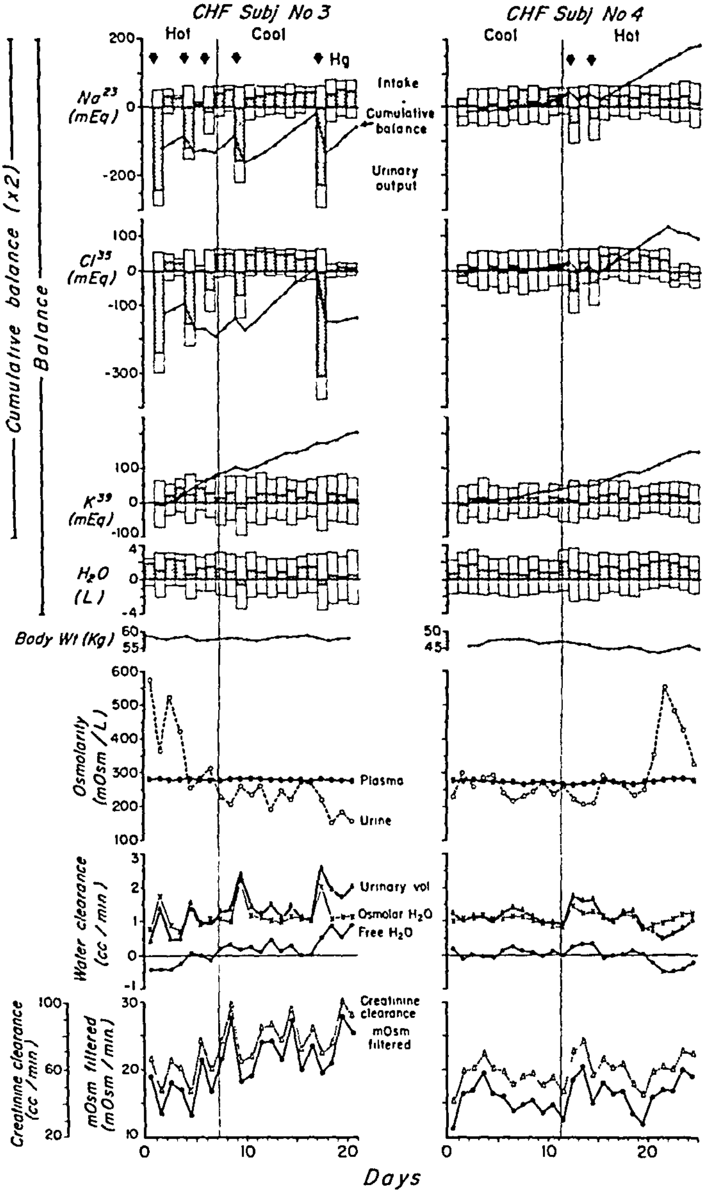

Fig. 6.—Time course of urinary excretion of water in 2 subjects with congestive heart failure. See legend for Fig. 5.

The time-course curves of average daily free water, osmolar water, and total water clearance rates expressed in milliliters per minute show considerable variation among the 8 subjects whose urinary collections were complete. Subjects 1 and 9 (Figs. 5 and 8) had free water excretion throughout the entire observation.

The effect of a mercurial diuretic on free water excretion was variable. The response to mercurials was not influenced by administration of a diuretic during periods when there was either positive or negative free water clearances.

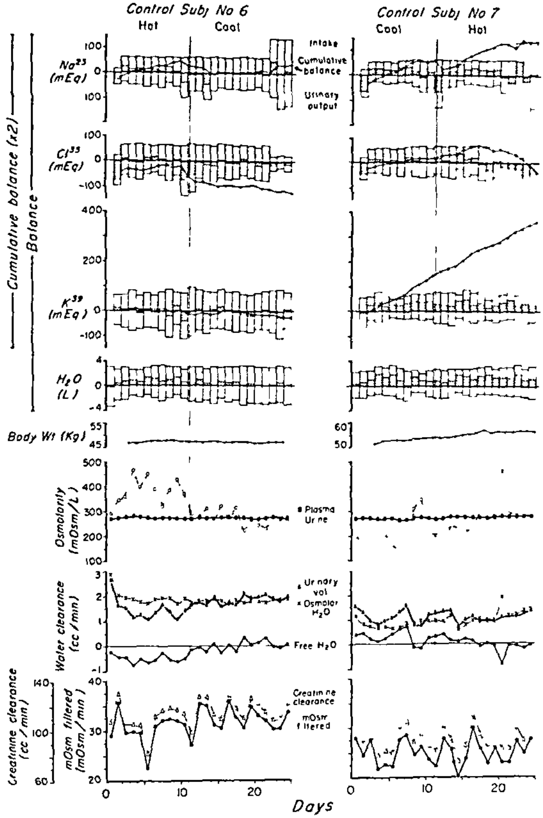

Fig. 7.—Time course of urinary excretion of water in 2 control subjects. See legend for Fig 5

Comment: The relative constancy of plasma osmolarity throughout these studies was to be expected with adequate electrolyte and water intake. One severely ill subject in this study, not shown in these figures, became dehydrated in the hot and humid environment and the plasma osmolarity rose by 20 mOsm. per liter.

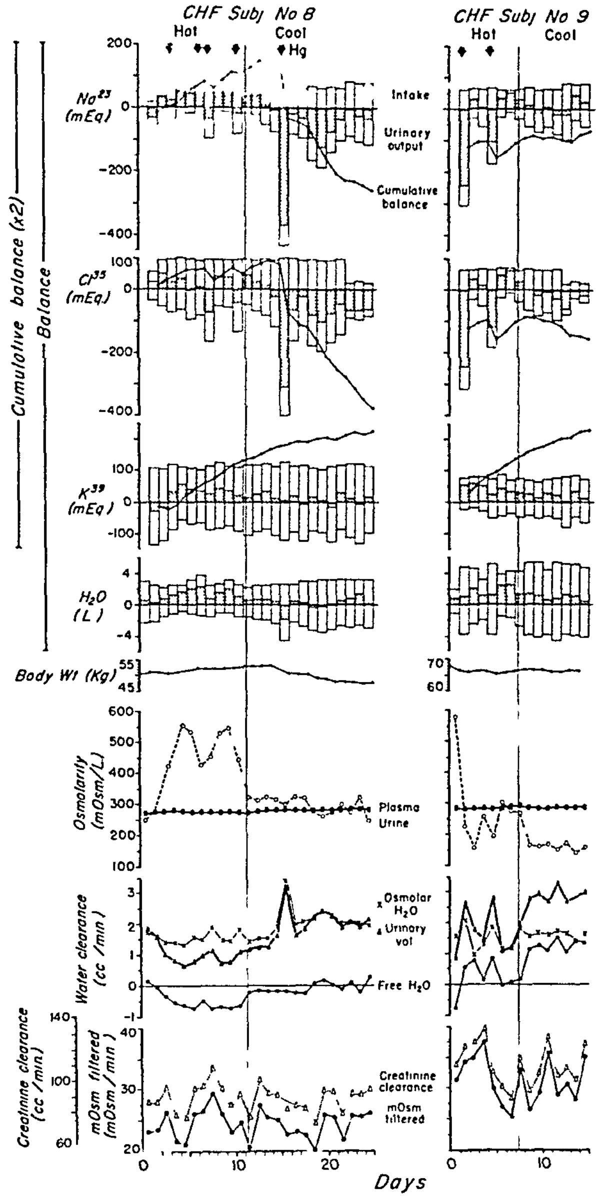

Fig. 8.—Time course of urinary excretion of water in 2 subjects with congestive heart failure See legend for Fig 5

The osmolarity of the urine in these subjects would be expected to vary according to the relative amounts of electrolyte and water intake which determines the amount to be excreted to maintain balance. With sweating, the osmolarity of the urine would be expected to be greater in the hot environment than in the cool one.

Free water, and thus total water excretion, reflected the difference between intake and nonurinary losses by respiratory tract and skin. Since these subjects were allowed water as desired, free water excretion reflected drinking habits, which kept two of the subjects in a state of constant water diuresis even in the hot and humid environment. The unusual drinking of water by Subject 9 when in the cool environment resulted in urinary volumes of approximately 4,000 ml. daily (Fig. 8). The remainder of the subjects had a daily water intake which resulted in a urinary osmolarity approximately equal to the plasma so that there was little free water clearance. The urine in Control Subject 6 and Subject 8 with heart failure was hyperosmolar to plasma with a $T^c_{H_2O}$ value of about 0.5 ml. per minute while in the hot and humid environment. Some of the subjects with congestive heart failure had urine hyperosmolar to plasma before therapy for their failure had begun. The two subjects on prednisone had higher osmotic loads for urinary excretion than the other subjects as evidenced by the prolonged periods of high osmolar water excretion.

Glomerular filtration rate. The average daily glomerular filtration rate was measured by endogenous creatinine clearances. The time courses of glomerular filtration rates are shown in Figs. 5, 6, 7, and 8 and Table IV. There were definite daily variations in the same subject. These variations were not influenced by osmolar load, the environment, or mercurial or spontaneous diuresis nor were they related to magnitude of osmolar, free, or total water clearance rates. The average creatinine clearance was slightly higher in the cool environment (86.49 ml./min.) than in the hot and humid one (82.98 ml./min.). There were periods when the reverse was true in 3 of the 8 subjects. The rates of creatinine clearance were as large in the subjects with congestive heart failure as in the normal control ones. In addition, the subjects with heart failure showed less change in glomerular filtration rate in the two environments than did the controls.

The average rate of glomerular filtration of mOsm. per minute summarized in Figs. 5 to 8 and Table IV were derived from the creatinine clearance and the osmolarity of the plasma for the same time. The milliosmolar reabsorption was about 98.5 per cent of that excreted throughout the period of study. There was a slight increase in the milliosmoles excreted in the cool environment over the hot and humid one, but the difference amounted to only 49 mOsm. per day, an amount well within the expected loss by sweating. In each subject, the milliosmolar excretion tended to be fairly constant with larger amounts excreted during mercurial and spontaneous diuresis.

Comment. Of the 4 subjects with congestive heart failure, 3 were moderately severe. However, since their rates of glomerular filtration were as high as those of the control subjects, inadequate glomerular filtration was not involved in the production of the clinical syndrome of congestive heart failure in these subjects.

The daily variability of glomerular filtration rate in both control subjects and in the subjects with heart failure was quite impressive. This has been true for all substances measured in the urine in this laboratory.[5, 8, 9, 10] Moment-to-moment variations of this function were not obtained in these experiments as was done in other studies in the past. However, large day-to-day variations were noted even though the subjects were at bed rest and in a steady metabolic state. These findings indicate further the need for multiple and prolonged observations of renal functions in order to establish the range of normal and to assess the effects of various procedures on such renal phenomena.

The relative independence of the osmolar load excreted from osmolar filtration is evident in these data. Adjustments to the needs of balance of both water and electrolytes over a wide range of filtration rates, and under the circumstances of two quite different environments, must have been primarily tubular reactions.

DISCUSSION

These studies revealed several interesting urinary excretory phenomena. For example, a hot and humid summer environment produced in most of the subjects a significant increase in extrarenal losses of water and electrolytes even though the subjects were at bed rest. In addition, there was an increase in osmolarity of the urine with a tendency to negative free water clearance. An unusual voluntary intake of water developed in some people acclimatized to a hot and humid summer and continued while they were in the air-conditioned ward where extra requirements for water were not necessary. The habit of drinking large quantities of water appeared to us to be a manifestation of acclimatizing adjustments made by some people of Louisiana to the heat and humidity of the summer. Probably with a longer period of residence in the cool environment the intake would have declined to the usual levels. Furthermore, the high intake, although adequate for the hot and humid environment and assuring essentially an average normal urinary state, resulted in a dilute urine of large volume when continued in the cool environment.

These experiments revealed the marked fluctuations in urinary clearance and excretion of Cl^{36}, Cl^{35}, Na^{23}, Rb^{86}, K^{39}, H^{3}, and H_2O when intake of these substances and physical activities were relatively constant. These marked daily variations support further similar previous observations[5, 8-10] of marked fluctuations over shorter periods of time as well as for daily intervals. In view of such variations, it is necessary to exercise caution when observing renal function and urinary excretion with only a few such measurements.

The daily endogenous creatinine clearances recorded for 26 consecutive days revealed no significant differences between the time course and magnitude of the rates of glomerular creatinine clearance of the subjects with or without chronic congestive heart failure. The patients with heart failure had both remissions and exacerbations in their failure during the observation period. It was evident from these experiments that any variations in renal function associated with the congestive failure were primarily tubular. Furthermore, it would likewise appear that unless congestive failure is severe, glomerular filtration remains within normal limits, at least for these subjects with moderately severe chronic congestive heart failure.

These experiments reveal the facility with which renal function may be followed continuously and simultaneously with different radioactive isotopes. Although only 3 were employed in this study, a larger number could be used equally well by taking advantage of differences in rates of physical decay and energy and spectra of radiation. Volatilization[11] and other special physical and chemical characteristics of the element may increase further the number which may be employed. There are many advantages in observing several different tracers simultaneously in a given animal rather than tracing each separately.

Again, these experiments confirm physiologic data previously described in man[5, 10, 12] with and without chronic congestive heart failure for Cl^{36}, Cl^{38}, Na^{22}, Na^{24}, Rb^{86}, and K^{42} and add the simultaneously observed renal excretion of H^3OH. These experiments add support to already existing data, thus increasing the validity of the findings for man as a whole.

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THE PRESERVATION OF BLOOD FOR TRANSFUSION

III. MECHANISM OF ACTION OF CONTAINERS ON RED BLOOD CELLS

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It has been shown that red cells collected and stored as whole blood are differently affected by the nature of the containers, as evidenced by in vitro tests and posttransfusion survival studies.[1, 2] The purpose of this investigation is to gain information on the mechanism of this effect.

We may consider the effect of containers on the red cells as possibly due to: (1) substances leached from the containers; (2) the liquid-air interface; (3) the water repellence and other physical properties. In the experimental work here presented these factors were analyzed individually.

MATERIALS AND METHODS

For the study of the effect of leached substances, donors were bled from the same venipuncture in 2 to 4 separate containers. The blood specimens were collected in ACD solution, formula NIH-A, at room temperature; the containers with the blood were then placed in an ice water bath for one-half hour and then stored at 5° C. One of the containers for each donor was always a standard borosilicate glass bottle, as control; a similar bottle contained strips of the plastic in amounts equivalent to the surface of a bag normally in contact with the blood. Whole plastic bags were also used, as controls, whenever commercially available (plastics C and X).

In addition to testing the plastic material actually used in bags available commercially for storage of blood, samples of plastic material were obtained to test the effect of plasticizers, stabilizers, and mold-releasing agents.

Plastic material EA-2158, EA-2159, and EA-2160 consisted of samples of laboratory-prepared polyvinyl chloride sheeting* of the following composition:

	EA-2158	EA-2159	EA-2160
Geon 101 EP (polyvinyl chloride)	62	62	62
Plexol 201-W (distilled DOS)	38	—	—
Monoplex DOS	—	38	38
Calcium stearate	—	—	1
Harshaw's 9V-1	—	—	2

Plastic sample EA-2158 is made of polyvinyl chloride plasticized with a distilled grade of dioctyl sebacate: this represents a higher quality than the Monoplex DOS, a standard grade of plasticizer used in material EA-2159. In sample EA-2160, calcium stearate is added as a mold-releasing agent and Harshaw's 9V-1 as a stabilizer. This material is a zinc compound

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mixed or associated with alkyl aryl phosphite. The zinc content is 2.7 per cent. Zinc as a stabilizer is commonly used when low toxicity is one of the properties desired and is the only metal present in any appreciable quantity in this plastic. Glass bottles containing the ACD solution and the plastic strips were sterilized with steam for 30 minutes at 121° C. The method for the measurement of red cells survival by tagging with Cr^{51} and for the study of properties in vitro are described in a previous publication.[3]

For the study of the liquid air interface, two plastic bags containing 75 ml. of ACD solution were used. With sterile technique, 37 ml. of solution was removed from each bag, in one approximately 250 ml. of filtered air was introduced. A donor was bled of 500 ml. of blood, 250 ml. being introduced directly in each of the two bags. Blood was stored at 5° C. for 3 weeks.

For the study of the effect of immediate and prolonged contact blood was collected in containers of plastic, glass, and silicone coated glass.* Blood was either cooled and stored in the same container or transferred before cooling to a container of a different nature as indicated in each experiment.

RESULTS

Effect of Substances Leached From the Containers.—The first series of experimental studies was directed to investigate the effect on red cells of substances possibly leached from the plastic. Table I shows the effect of the various plastics on the red cells, as determined by in vitro studies. It will be noted that the addition of plastic X, obtained from commercially available bags, and, to a lesser extent, of plastic EA-2158 is accompanied by an increase in the amount of free plasma hemoglobin. A similar effect is noted when blood is stored in a container made with plastic X. Hemolysis in 0.63 per cent saline solution appears to progress somewhat more rapidly when plastic X is added.

It is noted that damage to red cells, evidenced by increased free plasma hemoglobin, is exerted by the plastic material with or without the presence of glass surfaces. The high concentration of free plasma hemoglobin occurring when blood is collected in unsuitable plastic has been noted previously.[2] Plastic C added to a glass bottle does not increase significantly the concentration of free hemoglobin. *All other data reveal no significant differences.*

In subsequent experiments posttransfusion survival studies of red cells tagged with Cr^{51} were carried out simultaneously with the in vitro studies. Some of the plastic materials used in the first experiment were added to glass containers, and for each of 9 units of blood an autotransfusion and a transfusion in a compatible recipient were done, after a period of storage of 21 days.

In vitro studies showed variations in the plasma hemoglobin and in the adenosinetriphosphate (ATP) content, suggesting that the addition of plastic EA 2158 caused some *damage to the red cells. Much clearer evidence of damage* is shown by the results of posttranfusion survival of the same units of blood (Fig. 1). Normal posttransfusion survival was noted with the addition of plastic EA 2159. Similar results were obtained with plastic EA 2160, except for a single unit of blood. The average survival, however, of patients transfused with the units of blood to which plastic EA 2158 was added is definitely shorter

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*All glass containers were of borosilicate and obtained from the Wheaton Glass Company, Vineland, N. J. Silicone 1107 was employed as obtained from Dow Corning Corporation, Midland, Mich. Emulsion 35 A obtained from the same corporation was also used.

TABLE I

BLOOD UNIT	CONTAINER USED	PLASMA HEMOGLOBIN (MG./100 ML.)				PER CENT HEMOLYSIS IN 0.63% NaCl				ATP (MG./100 ML. OF RED CELLS)				DEXTROSE CONSUMPTION (MG./DAY/ML./OF RED CELLS)	LACTIC ACID PRODUCTION (MG./DAY/ML./OF RED CELLS)
		3*	4	5	6	3	4	5	6	3	4	5	6	6	6
372	Glass bottle Control	65	141	231	380	4	5	9	15	6.59	7.11	--	5.91	0.107	0.104
	Glass bottle Plastic C	104	133	226	355	4	5	6	9	5.58	7.38	--	4.17	0.108	0.096
	Plastic bag C	63	99	185	312	3	4	7	9	6.87	8.16	--	4.53	0.129	0.125
374	Glass bottle Control	151	208	337	370	6	9	12	22	6.24	4.95	3.72	3.78	0.086	0.080
	Glass bottle Plastic X	232	440	680	1140	7	9	15	24	7.78	6.29	3.60	--	0.069	0.069
	Plastic bag X	158	369	810	1060	10	13	22	28	5.54	4.83	4.95	0.3	0.079	0.086
373	Glass bottle Control	124	161	297	652	3	7	15	21	8.28	4.2	2.85	2.21	0.095	0.086
	Glass bottle Plastic EA-2158	145	192	290	484	4	7	11	21	7.71	4.11	3.66	3.21	0.108	0.078
379	Glass bottle Control	72	102	159	400	4	2	8	13	7.5	6.69	2.97	2.76	0.076	0.102
	Glass bottle Plastic EA-2158	116	150	265	355	4	2	7	9	7.32	3.81	3.93	3.77	0.070	0.103
	Glass bottle Plastic EA-2159	88	140	234	300	4	3	7	11	6.80	5.58	3.66	3.75	0.092	0.106
	Glass bottle Plastic EA-2160	50	91	146	229	4	2	5	8	9.02	4.59	3.93	3.26	0.083	0.100

*Numbers in subcolumns indicate weeks of storage.

For each unit of blood, designated by a number in the left-hand column, the results obtained with plastics must be evaluated by comparison of the data observed with the plain control glass bottles at various periods of storage, from 3 to 6 weeks.

than the average span observed for the units of blood stored in bottles to which other plastics were added. This evidence again suggests that some substance with a toxic effect on the red cells has been leached out of plastic EA 2158. The damaging effect of some commercially available plastic bags on red cells has been previously reported[1, 2].

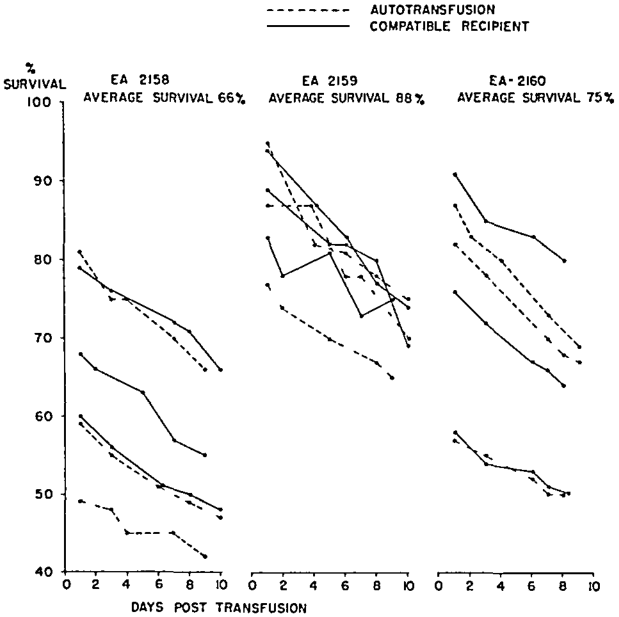

Fig. 1.—Posttransfusion Cr^{51} radioactivity survival of red cells stored as whole blood in borosilicate bottles to which various types of plastic material have been added.

Effect of the Liquid Air Interface.—Blood from a single donor was collected in two plastic bags, one containing filtered air and one collapsed; all other factors were similar. In Experiment 1 four recipients were transfused (Fig. 2). The 24 hour posttransfusion survival of radioactivity of Cr^{51} tagged red cells collected and stored in bags with the liquid air interface was 68 per cent; in the control series where blood was collected without liquid air interface, the survival was 67 per cent.

In the second experiment (Fig. 2) two recipients were used; at 24 hours posttransfusion the radioactivity survival of red cells collected and stored with the air liquid interface was 85 per cent; identical survival was noted for the red cells collected and stored without the air liquid interface.

TABLE II. RESULTS OF IN VITRO STUDIES ON BLOOD COLLECTED IN GLASS AND PLASTIC CONTAINERS, WITH LIQUID-AIR INTERFACE, AND SHAKEN AT DIFFERENT PERIODS OF STORAGE (EACH DATUM REPRESENTS THE AVERAGE OF DETERMINATIONS ON TWO SAMPLES)

		DAYS OF STORAGE	PLASMA HEMOGLOBIN (MG. %)	PER CENT HEMOLYSIS IN 0.63 % NaCl	ATP	DEXTROSE (MG. %)	LACTIC ACID (MG. %)	M.C.V. OF RED CELLS	pH
Shaking immediately after collection	Plastic containers	22	16	3	6.89	277	101	100	6.65
	Glass containers	21	16	5	7.08	270	102	100	6.65
Shaking after 10 days' storage	Plastic containers	22	42	1	6.22	272	130	99	6.64
	Glass containers	21	45	4	7.04	285	118	98	6.64
Shaking after 20 to 22 days' storage	Plastic containers	22	44	6	6.75	230	146	97	6.65
	Glass containers	21	71	8	6.25	247	125	98	6.70

In another experiment shaking was added to intensify the effects of the liquid air interface in the presence of wettable and water repelling surfaces Blood (500 ml) was collected from young, healthy donors into a plastic bag containing 75 ml of ACD solution The collection was made through plastic tubing, at room temperature and with hand mixing In each case, immediately after collection, 250 ml of blood was transferred to a standard glass donor bottle by gravity The plastic bag was then placed in a rigid container and partially filled with air Thus, the liquid air interface and the resiliency factors were practically eliminated, and the only remaining variable was the material of the container

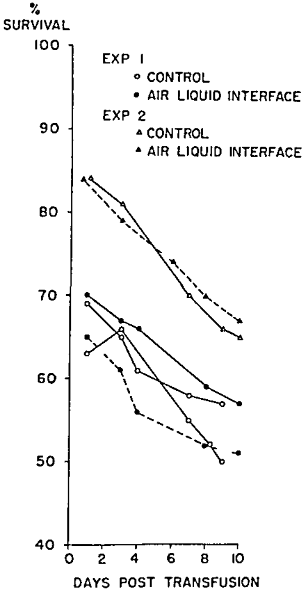

Fig 2—Posttransfusion Cr^{51} radioactivity survival of red cells stored as whole blood in plastic bags with air liquid interface and in control plastic bags without the air liquid interface

Shaking was carried out on a mechanical agitator for about one hour, at the following intervals of time (1) immediately after collection, (2) on the tenth day of storage, (3) after 20 to 22 days of storage, immediately before transfusion

All blood specimens were stored for 20 to 22 days and the red cells were tagged with Cr^{51} before transfusion

In vitro studies, immediately before transfusion, included plasma hemoglobin concentration, hemolysis of red cells in 0.63 per cent NaCl solution, A.T.P. content and mean corpuscular volume of red cells, dextrose and lactic acid concentration, and pH of plasma. No differences were found in any of the results which can be attributed to shaking or to the type of container in which the blood specimens were collected and stored for 20 to 22 days (Table II).

The results of the posttransfusion survival of red cells show that shaking of blood damages the red cells; no significant difference was found between the survival of red cells shaken in the two types of container (see Table III).

TABLE III. SURVIVAL OF Cr^{51} RADIOACTIVITY OF RED CELLS COLLECTED IN GLASS AND PLASTIC CONTAINERS, WITH LIQUID-AIR INTERFACE, SHAKEN AT DIFFERENT INTERVALS OF TIME, AND TRANSFUSED AFTER A STORAGE OF 20 TO 22 DAYS

	PLASTIC CONTAINERS		GLASS CONTAINERS	
TIME OF SHAKING	DAYS OF STORAGE	PER CENT RADIOACTIVITY 24 HR. POST-TRANSFUSION	DAYS OF STORAGE	PER CENT RADIOACTIVITY 24 HR. POST-TRANSFUSION
Immediately after collection	22	84	21	78
	22	83	21	76
	22	74	21	75
Average		80		76
At 10 days after collection	22	69	21	73
	22	71	21	69
	21	68	20	73
	21	74	20	69
Average		71		71
At 20 to 22 days after collection and immediately before transfusion	21	71	20	70
	21	67	20	70
	22	76	21	75
	22	72		
Average		72		72
Controls, not shaken, collected and stored in the same container				
	PLASTIC CONTAINERS		GLASS BOTTLES	
	21	89	21	63
	21	86	21	65
	21	84	22	93
	21	72	22	80
	21	87	20	70
	21	83	21	67
	21	93	21	53
	21	94	21	57
	21	82		
	21	82		
Average		85		69

Effect of Water Repellence and Other Physical Properties.—There has been much speculation concerning the effect of wettable and water-repellent surfaces on red cells, probably suggested by the effect noted on platelets.[4] The improved preservation of red cells stored in plastic containers has been attributed to the water repelling properties of plastic.[5] The results of the experiments previously reported on the effect of the liquid-air interface cast much doubt on the validity of this hypothesis. Agitation of blood in plastic and glass containers resulted in similar changes. More specific studies were carried out to determine the effect of wettable and water-repellent surfaces on red cells taking in consideration possible immediate effect and the effect of prolonged contact.

In the first experiment blood from 44 normal young men was collected with ACD solution as follows: in plain glass containers, 19 subjects; in silicone coated glass containers, 14 subjects; and in plastic containers, 11 subjects. The blood specimens, about 30 ml. each, were immediately tagged with Cr^{51} and autotransfused within one hour of collection. The results of study of posttransfusion survival are shown in Table IV. No significant difference is shown attributable to the immediate effect on red cells of the 3 types of containers, the mean survival at 24 hours posttransfusion for the 3 types being similar.

TABLE IV. EFFECT OF IMMEDIATE CONTACT WITH VARIOUS TYPES OF SURFACES ON THE POSTTRANSFUSION SURVIVAL OF RADIOACTIVITY OF Cr^{51} TAGGED RED CELLS, AUTOTRANSFUSED WITHIN ONE HOUR OF COLLECTION

TYPE OF CONTAINER	PER CENT SURVIVAL OF Cr^{51} RADIOACTIVITY 24 HR. POSTTRANSFUSION	MEAN
Borosilicate glass containers	99, 97, 97, 97, 96, 95, 94, 94, 94, 94, 94, 94, 92, 90, 90, 89, 89, 89, 87	93
Silicone coated glass containers	95, 95, 94, 94, 94, 93, 93, 92, 92, 92, 89, 89, 88, 87	92
Plastic containers	99, 96, 95, 95, 94, 94, 94, 92, 90, 89, 89	93
	Mean of 44 values = 93 Standard deviation = 3.1	

The effect of prolonged contact of red cells with different types of containers was studied in two groups in the following manner:

1. Blood was collected in plastic containers and immediately transferred to plain borosilicate bottles. In these experiments there were 3 donors and 3 transfusions, of which 2 were autotransfusions. The blood was stored from 21 to 22 days.

2. Blood was collected in glass bottles and immediately transferred to plastic bags. In this experiment 3 donors were used and the blood was autotransfused after 20 to 23 days of storage. In all instances, both donors and recipients were normal young men.

The results of posttransfusion survival studies are shown in Fig. 3. The blood collected in plastic containers and stored in glass containers shows a survival similar to the average obtained in 8 control units of normal blood collected and stored in glass containers for 21 days. The blood collected in glass containers and stored in plastic containers shows a survival similar to that obtained in 10 control units of normal blood collected and stored in plastic containers for 21 days.

Six units of blood from normal young men were collected in glass containers coated with silicone. The blood was stored in the same containers for 21 to 22 days, 4 units were autotransfused and 2 units were transfused in a compatible recipient.

The results are shown in Fig. 4. The average survival at 24 hours posttransfusion is 78 per cent, definitely higher than the average of a control group of 8 experiments using blood collected and stored in plain glass bottles for the same length of time. The survival values vary considerably in the 6 observations—from 68 to 92 per cent. The result with silicone coated glass surfaces

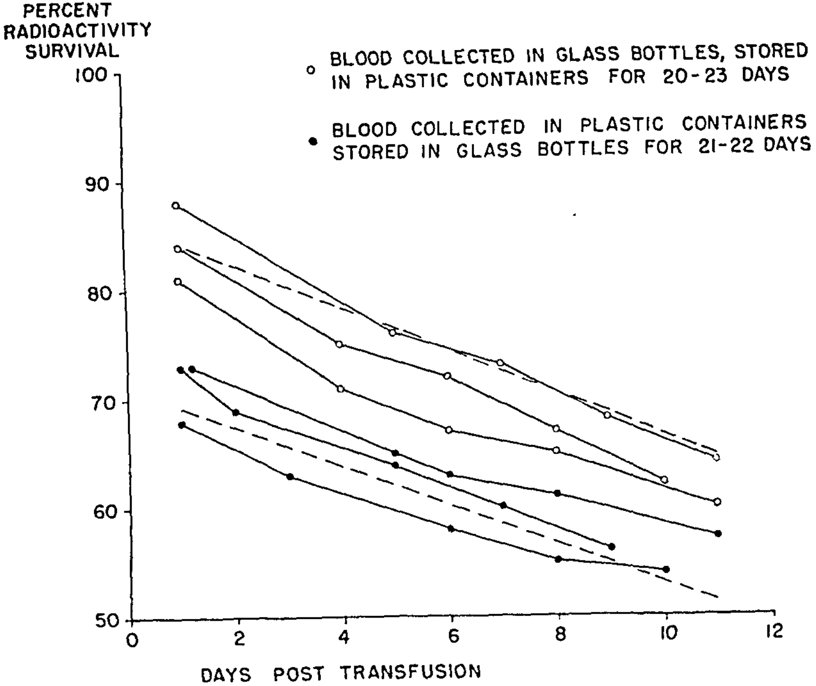

Fig. 3.

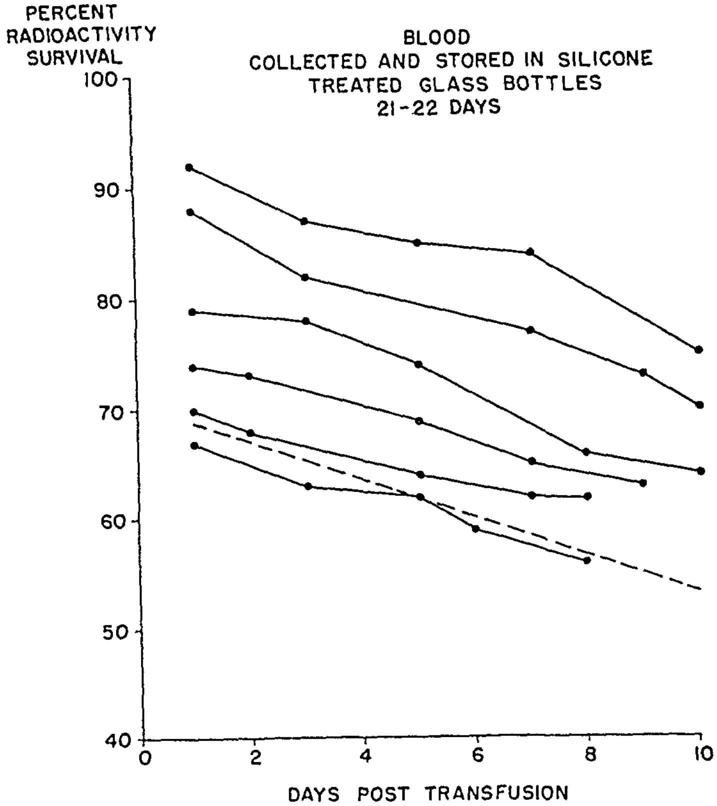

Fig. 4.

Fig. 3.—The upper interrupted line represents the average of Cr^{51} radioactivity survival of red cells in 10 transfusions of normal blood collected and stored in plastic containers for 21 days. The lower interrupted line represents the average of Cr^{51} radioactivity survival of red cells in 8 transfusions of normal blood collected and stored in glass containers for 21 days.

Fig. 4.—The interrupted line represents the average survival of red cells of 8 transfusions of normal blood collected and stored in glass containers for 21 days.

does not appear to be as good as the result obtained with the best types of plastic containers which, at various times have given a 24 hour posttransfusion survival of 84 to 87 per cent for red cells stored for 21 days

DISCUSSION AND SUMMARY

Experimental evidence has been presented supporting the view that certain materials employed in the preparation of plastic containers for collection and storage of blood exert a toxic effect on the red cells So far, polyvinyl chloride has been the only material used for the manufacture of containers for collection and storage of blood Even high grade polyvinyl chloride plasticized with a distilled grade of dioctyl sebacate may exert a toxic effect on red cells This toxic effect may be eliminated when the basic plastic is compounded by the addition of a proper stabilizer, such as one containing zinc Some plastic materials exert a toxic effect on red cells even after compounding with a stabilizer

It has also been shown that the harmful effect of the plastic material is due to leachable substance or substances, this unfavorable effect on red cells may be detected by in vitro studies These in vitro studies are recommended as screening tests for the evaluation of plastic materials intended for use in the manufacturing of containers for collection and storage of blood In vivo studies however, should always be used before releasing the containers for clinical use

The improvement which is noted on the red cell preservation when blood is stored in glass containers treated with silicone would indicate that glass containers also release substances toxic to the red cells When blood is collected in glass bottles containing ACD solution and the blood is removed from the bottle immediately after collection and transferred to a plastic container for storage, the properties of the ACD blood are similar to those of blood collected and stored in a plastic container This indicates that the toxic material released by the glass is not pre existent in the anticoagulant, but is formed from the interaction of citrated blood and container as the storage progresses This phenomenon is probably related to the solubility of silica At pH 5 of the ACD solution, silica is practically insoluble At pH 6 2, the solubility of silica is 0 2 parts per million At pH 7 of the ACD blood at time of collection, the solubility is 2 parts per million[6] It is probable that silicates leaching out from the glass during storage of blood are responsible for the damage to red cells, reducing their posttransfusion survival A similar conclusion applies to the effect of plastic material the toxic effect manifests itself only on prolonged contact during storage

It has been shown that the presence of the liquid air interface does not influence the in vitro properties and the posttransfusion survival of red cells No evidence has been found supporting the view that the surface properties of plastics have a causative relationship with the improved preservation of blood stored in some plastic containers Particularly suggestive are the results of shaking of red cells in the presence of wettable and nonwettable surfaces represented, respectively, by glass and plastic surfaces This experiment shows that shaking of blood immediately after collection is significantly less damaging to red cells than when done after a period of storage. This observation condemns the common practice of transporting blood after a period of storage.

The 24-hour posttransfusion survival of blood collected and stored in glass containers for 21 days has varied in experiments conducted at various times over a period of years from a mean of 69 to a mean of 74 per cent. These differences in results may be attributed to changes in the glass, probably due to reuse of the containers. Similar variations have been noted in the posttransfusion survival of blood collected and stored in glass containers coated with silicone: *these variations may be due to our inability to obtain a constant and* uniform coating of the glass surfaces.

CONCLUSION

The variable toxic effects noted on blood stored in glass and plastic containers is attributable to substances leached from the material of which the containers are made, either glass or plastic.

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IN VITRO STUDIES ON THE PRESERVATION OF MARROW CELLS IN GLYCEROL AT LOW TEMPERATURES

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WITH THE TECHNICAL ASSISTANCE OF LYLE NORTON, MARION WALES, AND DOROTHY THOMAS

IN LOWER animals, intravenous administration of marrow cells from hematologically normal donors will repopulate the marrow spaces and induce recovery after otherwise lethal exposure to ionizing radiation[1-4] In man marrow from fetal and adult cadavers and from biopsy and surgical specimens has been administered without untoward event and, at times has seemed to be of benefit to patients receiving large doses of total body radiation[5]

The large quantities of normal marrow required by clinical studies may be obtained by prompt sterile dissection of hematologically normal cadavers[6] Since suitable cadavers are not always available at the particular time when marrow is needed, some method for preserving marrow is desirable Successful preservation allows time for testing sterility, viability, and compatibility in homologous transfers and is essential when autologous marrow is used in patients receiving extensive radiotherapy or chemotherapy

It has been shown that mouse marrow retains its viability at 4° C for only 3 to 5 days[7] and for only about two weeks in tissue culture[8] Hematopoietic tissue of the mouse[9-11] and rabbit[12] have been preserved for long periods by freezing in glycerol to −79° C In these studies on rodents evidence of cell viability after preservation is obtained by demonstrating that intravenous administration of the preserved material prevents death after an otherwise lethal dose of radiation In vivo assay of this type is not often practical in man, though it has been accomplished in at least one instance[5] In vitro testing, therefore, must be used to provide preliminary information about preservation procedures applied to human marrow

This report describes in vitro studies of the effects of glycerol and freezing on human and rabbit marrow cells Cell counts, morphology, motility, dye permeability, and desoxyribonucleic acid (DNA) synthesis have all been used as criteria of preservation and viability Because the synthesis of DNA is a necessary preliminary to cell division, measurement of the rate of its synthesis has particular value as an indicator of proliferative ability[13]

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MATERIALS AND METHODS

Human marrow was expressed from ribs obtained at surgery or from postmortem dissections performed within 1 to 3 hours of death. From 0.5 to 2.5 billion nucleated cells were obtained from each rib. Rabbit marrow was obtained from healthy young adult animals. The marrow was suspended in 5 per cent autologous serum or human albumin in balanced salt solution (Hanks' solution)* and was passed through stainless steel screens to break up marrow particles.[14] The suspension was centrifuged at 200 g for 10 minutes, the supernatant fat and liquid were discarded, and the sedimented cells were resuspended in serum adjusted to a pH of 7.6 or tissue culture media and glycerol-saline as indicated in the individual experiments. One to five milliliter aliquots containing 10 to 100 million nucleated cells were pipetted into 10 ml. siliconed vials for assessment of freezing procedures.

Freezing was usually carried out by placing the vials in air at -20° C. for 25 minutes. Vials were then transferred to a dry beaker floating in a Dry Ice and alcohol bath at -79° C. for 15 minutes. The frozen vials were then stored at Dry Ice temperature, -79° C. This method of "slow" freezing was found to produce a temperature curve approximating the curve described by Barnes and Loutit,[9] i.e., 1 degree per minute to -15° C., then 2 degrees per minute to -70° C. "Rapid" freezing was achieved by immersing the vials in a Dry Ice and alcohol mixture and rotating them until freezing was completed. Thawing was carried out rapidly in a 37.5° C. water bath, and glycerol was removed by a modification of the technique recommended by Sloviter.[15] This consisted of adding one half volume of 35 per cent glucose in water to the cell suspension. One volume of saline was then added, followed by another volume of saline 5 minutes later, total dilution being 1 to 6. This diluted suspension was centrifuged at 200 g for 10 minutes and the supernatant discarded. The cells were resuspended in a 50 per cent serum and Hanks' solution and incubated for one hour in the presence of C^{14} formate. The incorporation of labeled formate into the thymine of DNA was determined as previously described.[13] Control aliquots of marrow not exposed to glycerol or freezing were similarly incubated as soon as possible after removal from the body in order to obtain a base-line measurement of normal activity.

Cell counting was performed under the phase microscope using 5 per cent albumin in Hanks' as the diluting fluid. Motility was observed under the phase microscope. Smears were made on cover slips from suspensions of the marrow in 5 per cent albumin in saline. On a number of occasions viability was assayed by the method of Schreck[16] using eosin. Samples considered nonviable by eosin staining were nonviable by other tests. However, some samples considered viable by the eosin technique were inactive when tested for DNA synthesis.

RESULTS

The Effect of Freezing in Glycerol on Cell Counts, Motility, and Morphology.—Marrow cells exposed to glycerol and freezing have been found to be extremely fragile. Initially it was found that after removal of the glycerol the cells tended to stick together in tenacious, stringy aggregates, making cell counts and morphologic observations impossible. After a few cells are disrupted by manipulation or preservation procedures, a sticky material appears in the suspending medium. This material presumably is DNA.[17] Formation of cell aggregates in this viscous material is prevented by adding recrystallized desoxyribonuclease† to the suspending medium in a concentration of 0.01 mg. per milliliter. This purified desoxyribonuclease was found not to inhibit DNA synthesis by marrow cells even in concentrations of 1 mg. per milliliter. Varidase* was found to inhibit DNA synthesis in concentrations of as little as 0.001 mg. per milliliter.

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*Sodium chloride 8.0 Gm., potassium chloride 0.4 Gm., magnesium sulfate 0.2 Gm., calcium chloride 0.14 Gm., sodium phosphate 0.06 Gm., potassium phosphate 0.06 Gm., and glucose 2.0 Gm. Sufficient distilled water to make 1 liter, pH is adjusted to 7.6 with 0.3 N sodium hydroxide.

†Worthington Biochemical Laboratory, Freehold, N. J.

After freezing in glycerol and thawing, marrow cells were found to be easily destroyed by the usual counting procedure. Exposure to 3 per cent acetic acid resulted in cell lysis or the formation of clumps that made counting impossible. Cells incubated in vitro for one or more hours at 37° C. (Table I) are readily destroyed by ordinary counting procedures using 3 per cent acetic acid. Initially this phenomenon gave rise to the erroneous impression that cells were being destroyed by the preservation procedure. Five per cent albumin in Hanks' solution, therefore, was used as the diluting fluid and counts were made under the phase microscope. It was then found that over 90 per cent of the nucleated marrow cells survived the glycerol and low temperature freezing procedure.

TABLE I. THE EFFECT OF THE DILUTING FLUID ON COUNTS OF HUMAN MARROW CELLS EXPOSED TO GLYCEROL AND FREEZING

DILUTING FLUID	NUCLEATED CELL COUNTS		
	INITIAL	1 HR	4 HR
Control			
3% acetic acid	27,750	24,100	23,800
5% albumin in Hank's solution	22,330	22,430	21,333
Glycerol and Freezing			
3% acetic acid	25,650	8,500	6,600
5% albumin in Hank's solution	21,133	20,367	20,333

The exaggerated mechanical fragility of preserved cells was also troublesome in making smears. Careful cover slip smears uniformly demonstrated smashing of the majority of the cells. When the smears were made using cells suspended in 5 per cent albumin in saline as described by Berenbaum[18] it was found that the cells were morphologically normal (Fig. 1).

Marrow cells subjected to the glycerol and low temperature procedure and subsequently protected against mechanical trauma and clumping by desoxyribonuclease, were found under the phase microscope to be normal morphologically. They exhibited motility of approximately the same degree as the control preparations.

The Effect of "Thermal Shock" and Storage Temperature on DNA Synthesis.—In studies on the preservation of bull semen,[19] sudden chilling has been found to be lethal. Fig. 2 illustrates an experiment designed to determine if sudden chilling to 0° C. is also detrimental to marrow cells. Only slight inhibition of DNA synthesis was observed in aliquots of human marrow exposed to a single sudden chilling, but repeated chilling depressed DNA synthesis appreciably. It appears that marrow cells should not be exposed to frequent changes of temperature during in vitro manipulations.

Human marrow and rabbit marrow, suspended in 5 per cent homologous serum Hanks' solution and stored at 4° C., maintain normal ability to synthesize

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*Streptokinase-streptodornase, Lederle Laboratories, Pearl River, N. Y.

DNA for approximately 24 hours. Viability is rapidly lost at room temperature, at 37° C., and on storage at 4° C. in acid citrate dextrose solution (Formula A, U.S.P.).

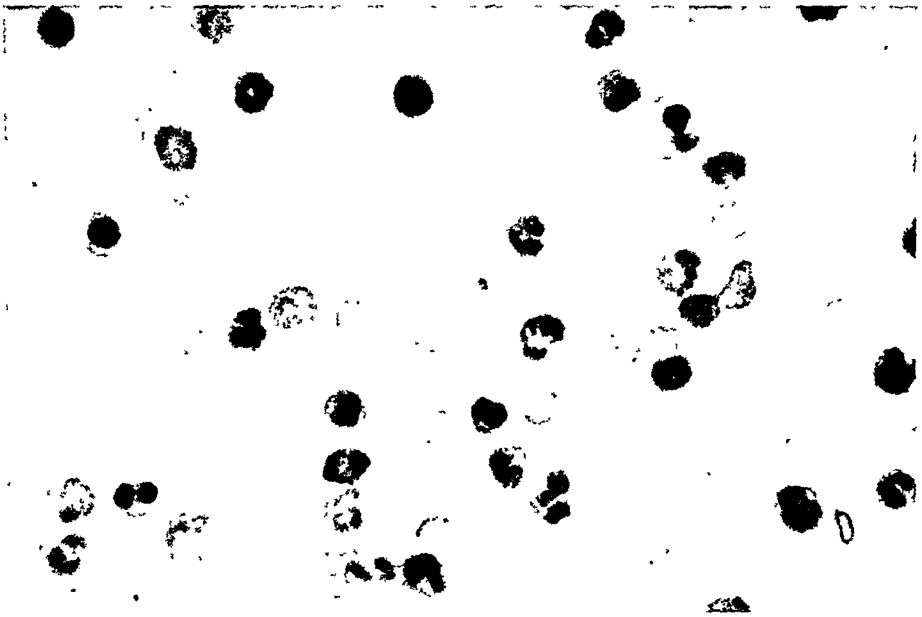

Fig. 1.—Human marrow after exposure to glycerol and freezing. Wright's stain (Magnification ×560, reduced ¼).

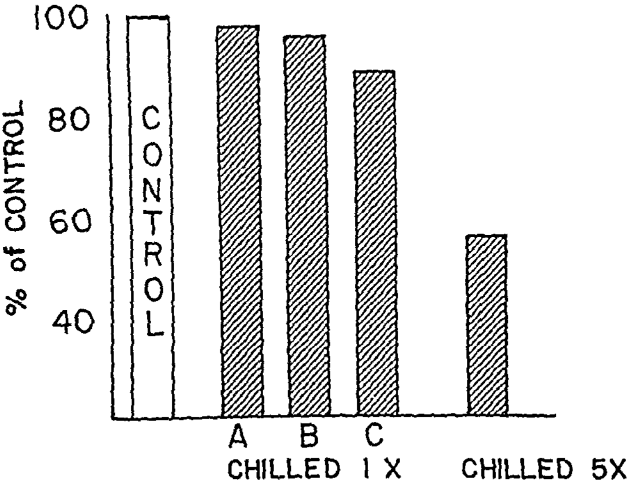

Fig. 2.—The effect of sudden chilling on DNA synthesis by human marrow cells.

The Effect of Glycerol on DNA Synthesis.—Fig. 3 illustrates an experiment designed to determine the effect of glycerol on the ability of marrow cells to synthesize DNA in vitro. It can be seen that all concentrations of glycerol in excess of 2.5 per cent depressed DNA synthesis. This experiment indicates the necessity of removing glycerol from cells before measuring DNA synthesis.

In 4 experiments (Table II) the ability of marrow cells to synthesize DNA after exposure to 15 per cent glycerol without freezing was determined and compared with similar cells after exposure to both freezing and glycerol. It is apparent that much of the trauma of the preservation procedure is due to the exposure to glycerol.

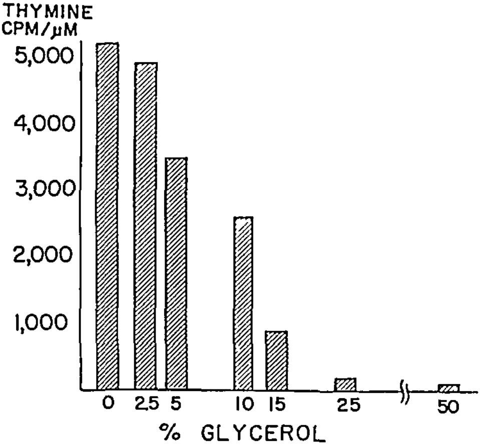

Fig. 3.—The effect of glycerol concentration on DNA synthesis by rabbit marrow.

TABLE II. A COMPARISON OF THE EFFECT OF GLYCEROL ALONE AND GLYCEROL PLUS FREEZING ON THE SYNTHESIS OF DNA BY MARROW CELLS (PER CENT OF CONTROL)

MARROW SOURCE	GLYCEROL (PER CENT)	GLYCEROL AND FREEZING (PER CENT)
Human	40	40
Human	72	53
Human	58	28
Rabbit	66	49

The Effect of Glycerol and Freezing on DNA Synthesis.—Glycerol low temperature preservation of the ability of marrow cells to synthesize DNA is illustrated in Fig. 4 showing data from 11 experiments on human marrow and 7 experiments on rabbit marrow. A wide range of degree of preservation is evident.

Fig. 5 shows the results of 10 experiments in which preservation of ability to synthesize DNA was correlated with the percentage of erythroid cells in the marrow. A close correlation between degree of preservation and percentage erythroid elements is evident, indicating that DNA synthesis after

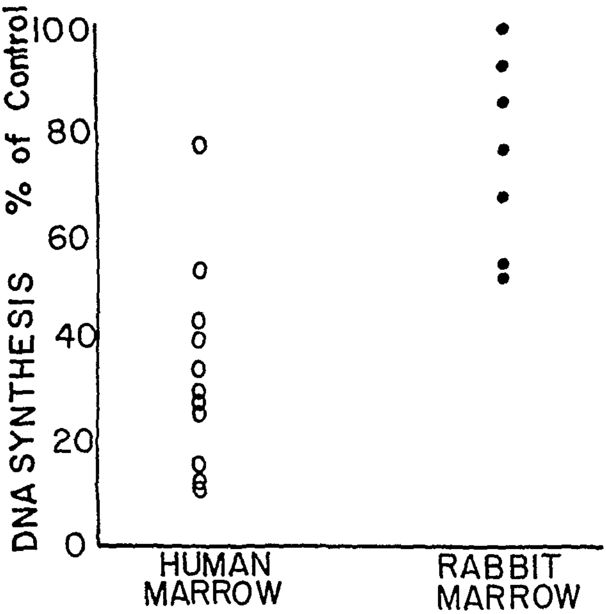

Fig. 4.—The ability of marrow cells to synthesize DNA after preservation in glycerol at -79° C.

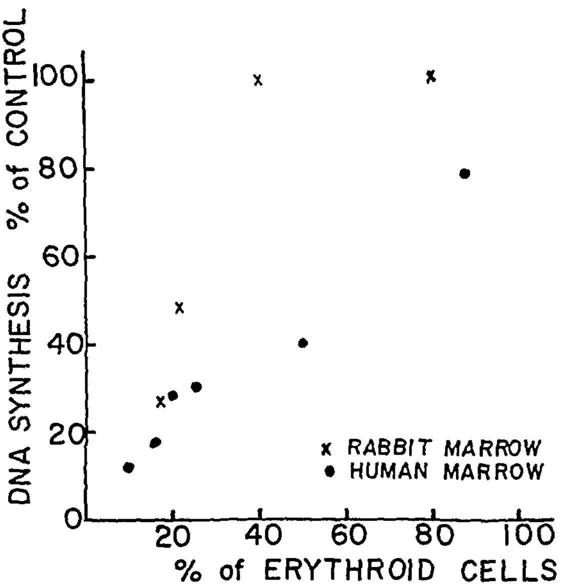

Fig. 5.—The correlation between the preservation of the ability to synthesize DNA and the percentage of erythroid elements in the marrow.

low-temperature preservation is preferentially maintained in erythroid cells This observation may account for the variability of degree of preservation presented in Fig 4

In 3 experiments with preserved rabbit marrow the DNA synthesis of cells originally suspended and frozen in 50 per cent serum glycerol was found to be twofold better than that of cells that had been suspended and frozen in 5 per cent serum glycerol This observation of a protective effect of high concentrations of serum during freezing in glycerol has not been confirmed with human marrow as yet

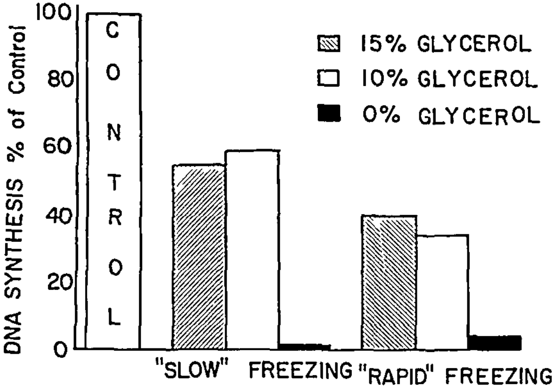

Fig 6—The effect of glycerol concentration and the rate of freezing on preservation of DNA synthesis by rabbit marrow

The Effect of Freezing Rate and Glycerol Concentration on Preservation of DNA Synthesis—Fig 6 illustrates one of several experiments with rabbit marrow designed to determine the importance of the rate of freezing and the concentration of glycerol on the preservation of marrow cells There is no significant difference between 10 and 15 per cent glycerol, but freezing without glycerol results in cells "nonviable" in our in vitro assays In general "slow" freezing permits better preservation than "rapid" freezing, but the difference is barely significant over the range studied

The Postmortem Effect on Preservation—Table III shows the results of 3 experiments with rabbit marrow designed to determine the effect of post mortem autolysis on marrow cell viability and marrow cell preservation In rabbit marrow a considerable decrease in ability to synthesize DNA is apparent after 2 hours of in situ postmortem autolysis at room temperature As indicated in Table III, rabbit marrow cells subjected to the trauma of in situ postmortem autolysis have very limited ability to withstand the further trauma of preservation with glycerol and low temperatures Some evidence has been presented to indicate that postmortem autolytic phenomena do not seriously impair DNA synthesis in the marrow of human cadavers. The general and specific implication of these studies is that the sooner cadaver marrow is harvested, the better.

TABLE III. THE EFFECT OF TIME AFTER DEATH ON DNA SYNTHESIS BY RABBIT MARROW

EXPERIMENT	HOURS POSTMORTEM	THYMINE (C.P.M./μM)	
		CONTROL	FROZEN
A	0	7,890	9,895
	2	839	190
B	0	6,490	6,975
	3	1,021	649
C	0	7,950	2,150
	3	149	15

DISCUSSION

The studies presented show that exposure of marrow cells to glycerol and subsequent removal of glycerol accounts for an appreciable fraction of the trauma associated with the preservation procedure of glycerol at low temperatures. Trauma may occur when the glycerol enters the cell, during its presence in the cell, or during its removal from the cell. Preliminary studies of the importance of rates of addition or removal of glycerol have led to no significant improvements in the procedure. Dialysis allows slow removal of the glycerol but the progressive deterioration of marrow cells suspended in glycerol at 4° C. makes this procedure undesirable. It is possible that the removal of glycerol may be found unnecessary in the clinical use of preserved marrow. Preserved rodent marrow can be given intravenously without removal of glycerol. Preserved mouse marrow so administered is unimpaired in its ability to induce recovery after lethal irradiation.[11]

Our data indicate a relatively small difference in viability of marrow preserved by "slow" freezing, 1 or 2 degrees per minute, and the viability of marrow by "rapid" or shell freezing. Because of the small difference observed over this wide range, we have not investigated intermediate rates of freezing. For the present it appears preferable to follow the "slow" rate. However, close adherence to this rate does not seem essential.

In a previous communication[5] we reported that human cadaver marrow exhibited approximately normal rates of DNA synthesis on in vitro assay after 2 to 6 hours of in situ postmortem changes. The studies on postmortem effects in rabbit marrow reported here indicate that in situ postmortem changes cause a significant and progressive loss of ability to synthesize DNA in vitro. This loss is aggravated by subsequent application of preservation procedures which are in themselves traumatic. Hence, if useful human cadaver marrow is to be stored, it would appear to be important to make every effort to obtain collections as soon after death as possible.

The data in Fig. 5, indicating that DNA synthesis in myeloid marrow is poorly preserved by freezing in glycerol, are disturbing. If these data are substantiated, they suggest that preserved marrow may be a relatively poor source of the myeloid elements necessary for early defense against infection in the irradiated individual. It is reassuring that rabbits protected against death from lethal irradiation by preserved marrow have shown adequate regeneration of the myeloid elements of their marrow.[1] Studies are in progress using autoradiographs with tritium labeled thymidine to secure direct evidence about the relative effect of preservation with glycerol at low temperatures upon the preservation of myeloid and erythroid marrow cells of man.

The wide variability in the degree of preservation of human marrow and the generally better preservation of rabbit marrow deserve comment. The rabbits studied were all young healthy animals, and their marrow was obtained immediately after death. The patients from whom ribs were obtained were in the older age group and were suffering from lung disease or heart disease. The proportion of myeloid cells was greater in the human marrows than in the rabbit marrows. Obviously, young healthy individuals make the best marrow donors. The large number of marrow cells obtainable at autopsy may compensate for some potentially disadvantageous aspects of specimens obtained from older and debilitated patients.

The data presented here indicate that human marrow cells can be preserved by freezing in glycerol at -79° C. After subsequent thawing and removal of the glycerol, these marrow cells remain intact, appear morphologically normal, demonstrate motility, and exhibit an appreciable fraction of their original ability to synthesize DNA. Rodent marrow treated in comparable fashion has been kept for 6 weeks at -79° C in glycerol and serum, and this marrow, despite impaired ability to synthesize DNA in vitro has shown unimpaired ability to proliferate and repopulate the marrow of an irradiated host.[11] Much of the cell damage associated with the low temperature glycerol preservation procedure is here demonstrated to be associated with the addition and the removal of glycerol. The important question is whether this damage is shared equally by all cells. The answer would appear to be that it is not. Myeloid elements suffer more than erythroid. How the essential "seed" cells make out remains to be determined. For solution of this critical problem one must turn to autoradiographs with tritiated thymidine. With the techniques described here, human marrow preserved for 8 weeks has shown no progressive deterioration. Accordingly, it seems logical to suppose that human marrow can be kept indefinitely with a useful maintenance of cells capable of proliferating in an irradiated host. The low temperature glycerol procedure, therefore is being continued as the basis for the current establishment of "banks" of frozen human marrow for autologous and homologous use.

SUMMARY

Recent interest in preservation of human marrow for both homologous and autologous use has indicated a need for evaluation of various preservation methods. This report describes in vitro studies of the effects of glycerol and freezing on human and rabbit marrow cells. Cell counts, morphology, motility, dye permeability, and DNA synthesis have been used as criteria of viability. When protected against mechanical and chemical trauma, and against clumping by the use of desoxyribonuclease, excellent morphologic preservation was achieved. DNA synthesis decreased after repeated chilling without freezing

Glycerol inhibited DNA synthesis in vitro, and marrow cells exposed to 15 per cent glycerol showed moderate impairment of their ability to synthesize DNA after removal of the glycerol. The freezing to −79° C. of marrow cells in 15 per cent glycerol resulted in a further moderate decrease in ability to synthesize DNA. Myeloid cells appeared to suffer greater damage from the freezing procedure than did erythroid cells. Limited observations on the rate of freezing and the concentration of glycerol indicate that these factors are not of critical importance. An early postmortem deterioration of rabbit marrow cells was demonstrated.

The data presented here indicate that human marrow cells can be preserved by freezing in glycerol at −79° C. Preserved marrow has shown no further deterioration after 8 weeks of storage. The glycerol low-temperature method is the procedure employed in the current establishment of "banks" of frozen human marrow.

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OBSERVATIONS ON THE SITE OF PRODUCTION OF CASTLE'S INTRINSIC FACTOR IN THE RAT

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In 1929, Castle demonstrated that the fundamental disturbance in pernicious anemia was the absence from gastric juice of a substance, which he called "intrinsic factor." The intrinsic factor is generally believed to play an essential part in the physiologic mechanism of the intestinal absorption of vitamin B_{12} (see review by Castle[1]).

A number of investigations has been performed on the site of production of the intrinsic factor, especially in the hog and in man. Recently the rat has proved to be a suitable experimental animal for investigations of this kind. Watson and Florey[2] demonstrated that removal of the glandular part of the stomach in the rat interferes with intestinal absorption of vitamin B_{12}, as shown by increased fecal excretion of orally administered radioactive vitamin B_{12}. Extracts of the glandular part of the gastric mucosa proved to have intrinsic factor activity, because on oral administration they restored vitamin B_{12} absorption to normal values. Similar results have been reported by Nieweg and associates.[3] In these latter experiments total gastrectomy was found to result in a high excretion of orally administered Co^{60} vitamin B_{12}. Both rat gastric juice and homogenates of total rat stomach wall or of the glandular part of the rat stomach, if administered to gastrectomized animals together with vitamin B_{12}, reduced excretion to the values found in normal animals.

In the biologic part of the present investigation the regional localization of intrinsic factor production in the rat stomach has been further studied in absorption experiments. First, the intestinal absorption of radioactive vitamin B_{12}, as measured by fecal excretion, was determined in rats in which different parts of the stomach had been separately removed. Subsequently, extracts of the different regions of the rat stomach wall were tested for intrinsic factor activity, measured as the capacity to reduce the fecal excretion of vitamin B_{12} in gastrectomized animals.

Concerning the cellular localization of Castle's intrinsic factor in the gastrointestinal tract, the evidence available so far is inconclusive. Jacobson[4] had suggested the argentaffine cells as a possible site of production. This view has been rejected by most investigators, because the regional distribution of these cells does not correspond to what is known about the occurrence of intrinsic factor. Glass and co-workers[5] isolated from human gastric juice a

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strongly acidic "glandular mucoprotein" secreted, according to them, by the mucoid neck cells and containing nearly all of the intrinsic factor activity of the starting material. This acidic glandular mucoprotein, in experiments of Crevier and Bélanger,[6] was shown to consist largely of sulfomucoprotein. This result was interpreted as indicating that 3 types of gastric cells, known to produce sulfomucoproteins (Bélanger[7]), would be responsible for intrinsic factor production. These cell types are: (a) the mucoid neck cells of the fundus glands, (b) the cells of the mucous glands in the pyloric region, and (c) the cells of some few mucous glands of the cardiac-fundus transitional zone. This argumentation, however, was largely invaluated by subsequent work of Cresseri[8, 9] and particularly Gräsbeck.[10, 11]

Cresseri demonstrated that, in glandular mucoprotein preparations from *hog gastric mucosa, all of the vitamin* B_{12} *binding capacity resided in a fraction* containing neutral polysaccharide coupled to a protein moiety; it was completely absent from the acidic mucoprotein fractions. Though not conclusive, due to the nonspecific character of vitamin B_{12} binding as such, this result still suggests that intrinsic factor might be contained in the neutral polysaccharide-protein components rather than in the acidic sulfomucoproteins. Gräsbeck has more recently demonstrated that intrinsic factor activity itself in human gastric juice was definitely not associated with acidic glandular mucoprotein if denaturation was avoided by immediate cooling of the freshly collected gastric juice. In these experiments the entire intrinsic factor activity was recovered from a slow-moving zone in starch block electrophoresis; the fast-moving acidic mucoids did not show any activity.

In the histologic part of the present investigation, two approaches to the problem of cellular localization of intrinsic factor activity were tried. Sections of rat gastric mucosa were studied (1) with the periodic acid-Schiff (PAS) technique and (2) through autoradiography with $Co^{56, 57}B_{12}$.

The PAS technique is considered to demonstrate the presence of neutral polysaccharides (polyhexoses) and was chosen in view of the neutral polysaccharide-protein nature of Cresseri's vitamin B_{12} binding preparation. The aim was to investigate a possible correlation between the occurrence of PAS positive cells in the gastric mucosa and the regional localization of intrinsic factor activity as indicated by the biologic part of the present investigation.

The autoradiographic procedure, using $Co^{56, 57}B_{12}$, would seem to promise more direct information concerning the cellular localization of intrinsic factor activity through its vitamin B_{12} binding capacity, if at least nonspecific vitamin B_{12} binding could be excluded.

MATERIALS AND METHODS

Male white rats weighing 250 to 300 grams were used in these studies. The radioactive vitamin B_{12} used was labeled with $Co^{56, 57}$. Its specific activity at the time of delivery varied from 2.4 to 11.7 μc per microgram for the different batches.

For the biologic experiments the animals were operated under pentobarbital sodium (Nembutal) anesthesia. When total gastrectomy was performed, an end-to-end anastomosis between esophagus and bisected pylorus was made. Partial gastrectomies were done on the guidance of the macroscopic appearance of the gastric mucosal regions. The proximal part of the rat stomach is covered with squamous epithelium and has a pale gray color. In the distal glandular part, the fundus region can be easily distinguished from the antrum pylori by its more pronounced pink aspect. In the first type of partial gastrectomy both the nonglandular fore-stomach and the fundus region were resected, while the pyloric region was left intact; in the second type the fore-stomach alone was removed. These partial gastrectomies proved to be technically more difficult than total resections; leaking of the suture line with subsequent peritonitis leading to death occurred more frequently.

Extracts of the different parts of the rat stomach were made by the procedure of Watson and Florey.[9]

During the absorption experiments the animals were kept in metabolic cages and given a stock diet and water ad libitum. They were fed a test dose of 5 mμg labeled vitamin B_{12} in combination with either 0.1 ml. of saline or 0.1 ml. of an extract of stomach wall. The stools were collected over a 4 day period. After drying the feces were weighed and ground to a fine powder. The radioactivity was measured with an end-window G.M. tube with a Philips P.W. 40 31 electronic counter. The results were expressed as percentages of the test dose of labeled vitamin B_{12}.

For the histologic work the animals were killed by a blow on the head. The stomach was immediately removed and opened along the greater curvature, stretched on a cork plate, and fixed in Bouin's solution, 3 per cent formalin or cold saturated ammonium sulfate. Afterward the material was trimmed so that the sections would contain the entire length of the lesser curvature, including small portions of the esophagus and duodenum; in addition, separate fragments of fundus mucosa were taken.

The PAS reaction was performed on paraffin sections of material fixed in either Bouin's solution or formalin; the PAS procedure of McManus[12] was used throughout. For counterstaining hematoxylin was used.

For the autoradiographic procedure material fixed in cold saturated ammonium sulfate was used. This fixative was chosen because it has been widely used in the chemical isolation of intrinsic factor, without impairing its biologic activity. Frozen sections were incubated for 3 hours with a solution of Co^{56} B_{12}, saturated with ammonium sulfate. The sections were subsequently washed in 3 portions of saturated ammonium sulfate, given a final short rinse of distilled water, and mounted on slides treated with gelatin chromalum. These slides were either placed in contact with radiographic dental film (Dozahn Schleusner) or covered with stripping film (from Kodak autoradiographic plates AR 10 and AR 50). After varying periods of exposure in the cold the material was developed.

The sections that had been in contact with dental film were stained with hematoxylin or PAS; the sections covered with stripping film were mounted unstained and viewed by phase contrast microscopy.

In addition a number of routine techniques were used as indicated in the text.

RESULTS

Absorption Studies.—In a first set of experiments the fecal excretion of Co^{56} $^{57}B_{12}$ was measured in rats in which either (a) the fore-stomach (cardiac region) alone or (b) the fore-stomach and the fundus region had been resected. Two groups of rats served as controls: normal animals and animals after total gastrectomy. In each experiment a single test dose of 5 mμg Co^{56} $^{57}B_{12}$ was orally administered followed by 0.1 ml. of saline. The results, presented in detail in Table I, can be summarized as follows.

After removal of the *fore-stomach* alone (a), 46 per cent of the vitamin B_{12} test dose was recovered in the stools (average of 6 experiments). This value does not differ significantly from that of the *normal* controls (12 experiments), in which 36 per cent of the vitamin B_{12} was excreted. It proves that the distal parts of the stomach produce sufficient quantities of intrinsic factor to maintain a nearly normal intestinal absorption of vitamin B_{12}.

Animals in which the *fore-stomach and the fundus region* had been resected (b) excreted an average of 70 per cent of the vitamin B_{12} test dose (6 experiments). This value compares well with the average excretion of 77 per cent in the total gastrectomy controls (11 experiments). It means that the antrum pylori, being unable to promote vitamin B_{12} absorption, fails to produce significant amounts of intrinsic factor.

These experiments together, therefore, suggest that the fundus region is either the main or the only site of intrinsic factor production.

In a second set of experiments, extracts of the fundus wall (c) and of the antrum pylori (d) were tested for intrinsic factor activity, i.e., for their capacity to restore vitamin B_{12} absorption in rats in which total gastrectomy had been performed. A control group of gastrectomized animals was treated with an extract of whole stomach wall. The results of these experiments are also recorded in Table I.

TABLE I. FECAL EXCRETION OF RADIOACTIVITY EXPRESSED AS A PERCENTAGE OF THE TEST DOSE OF $Co^{56,57}B_{12}$ IN NORMAL RATS AND IN RATS IN WHICH DIFFERENT TYPES OF GASTRECTOMIES HAVE BEEN PERFORMED

NORMAL ANIMALS	RESECTION OF FORE-STOMACH (a)	RESECTION OF FORE-STOMACH AND FUNDUS (b)	TOTAL GASTRECTOMY			
			SALINE CONTROLS	FUNDUS EXTRACT (c)	ANTRUM PYLORI EXTRACT (d)	WHOLE STOMACH EXTRACT
27	37	55	52	44	77	36
31	37	67	62	58	82	36
32	41	68	70		88	40
32	48	68	73			42
34	54	72	74			45
34	58	92	75			46
35			79			54
36			80			57
39			86.5			58
41			89			64
43			104			
48						
Mean 36 ± 5.6	46 ± 8.2	70 ± 11.0	77 ± 13.2	51 ± 7	82 ± 4.5	48 ± 9.3

It can be seen that fundus wall extract (c) reduced the vitamin B_{12} excretion of gastrectomized rats to 51 per cent (mean of 2 experiments). This value corresponds well to the 48 per cent found in the control group (10 experiments) treated with whole stomach wall extract and to the values found in normal animals.

In 3 experiments, in which an extract of antrum pylori was given (d), however, the average excretion of vitamin B_{12} was 82 per cent.

Previously published experiments[3] already have shown that homogenates of fore-stomach wall lacked intrinsic factor activity under similar conditions. Apparently the fundus wall is the only part of the rat stomach which contains intrinsic factor. The two sets of absorption experiments together therefore clearly *prove that in the rat the fundus region is the site of production of* Castle's intrinsic factor.

Histologic Studies.—In the PAS treated sections of the rat gastric mucosa abundant PAS positive material was present in the surface epithelium of both fundus and pyloric regions. To avoid misunderstanding it may be stated that "surface epithelium" means the lining of gastric areolae and gastric pits. The latter reach to nearly one third of the mucosal thickness in the fundus, and to nearly two thirds in the pyloric region.

As the fundus was the only region exhibiting intrinsic factor activity in the biologic experiments described above, its histology was of particular interest. Here, in addition to the surface epithelium, one cell type gave a positive PAS reaction: the mucoid neck cells of the fundus glands. These mucoid neck cells were found in a distinct zone approximately corresponding to the middle third of the mucosal thickness in the fundus. The lower third, occupied by the basophilic chief cells (pepsinogen cells), did not exhibit any noticeable PAS color. Both zones did, of course, contain oxyntic cells (hydrochloric acid cells); these latter cells, as usual, were somewhat more numerous in the neck cell zone.

The cardiac region of the rat stomach is lined with nonkeratinized stratified squamous epithelium, without any glandular structures. No PAS reactivity was observed in this epithelium.

The pyloric region contains the usual simple tubular glands which occupy only the lower third of the mucosal thickness. These glands, although clearly of mucous character, gave only a very weak PAS reaction.

In the adjacent duodenal mucosa only the solitary goblet cells and the submucosal Brunner's glands were PAS positive, the latter only weakly.

These results again pointed to the mucoid neck cells of the fundus glands as possibly being responsible for intrinsic factor production. In view of the strict localization zone of these cells in the fundus mucosa, it might be expected that an equally distinct zone of blackening would be found in the autoradiographs, that is, if mucoid neck cells did contain intrinsic factor.

The contact autoradiographs of histologic sections incubated with Co^{60} B_{12} gave a clear picture of the regional distribution of the radioactive material. This was especially the case in longitudinal sections covering the entire lesser curvature. In Fig. 1, the (dental film) contact autoradiograph (Fig. 1, *B*) of such a section is reproduced together with its stained mother section (Fig. 1, *A*). Only one distinct region has given a definite blackening in the autoradiograph: the fundus region of the gastric mucosa. To the left the stratified squamous epithelium of the cardiac region can be seen to be entirely without radioactivity. The same holds for the pyloric region to the right. Here, only some irregular spots of blackening are present, which are partly due to some folds in the section. The duodenal part of the section, to the extreme right, also did not produce any blackening, neither in the mucosa, nor in the submucosal Brunner's glands. This regional distribution of vitamin

B_{12} binding, as demonstrated autoradiographically, completely corresponds to the regional localization of intrinsic factor activity as shown by the absorption experiments described above.

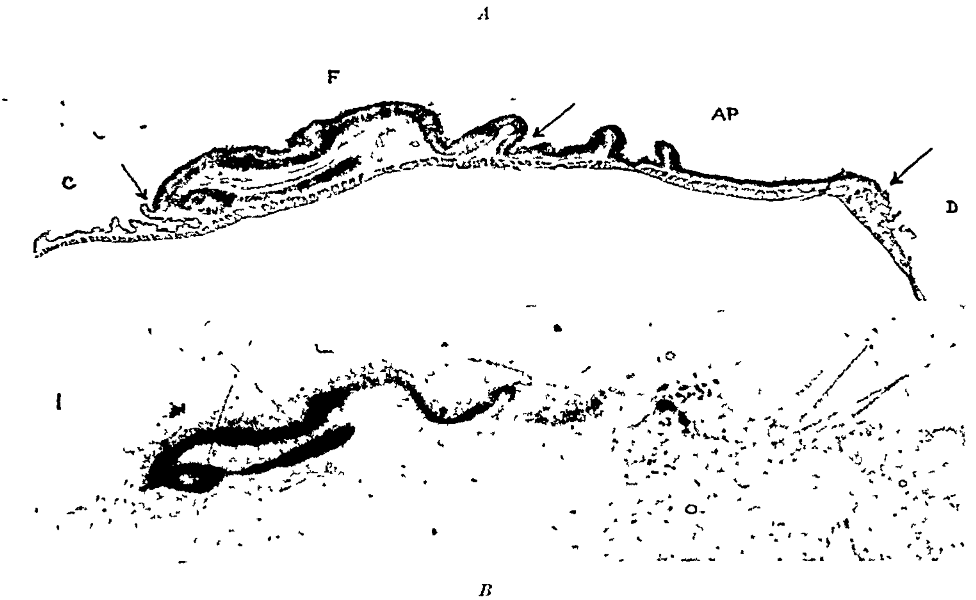

Fig. 1.—Longitudinal section of rat stomach along the small curvature, incubated with Co^{58} B_{12}. A, Mother section stained with hematoxylin. The arrows point to transitions of cardia (C) to fundus (F), fundus to antrum pylori (AP), and antrum pylori to duodenum (D), respectively. *B*, Dental film contact autoradiograph, exposure 2½ days. Vitamin B_{12} binding is only found in the fundus region.

More detailed information concerning the localization of the vitamin B_{12} binding sites in the fundus mucosa comes from the following pictures. Fig. 2, *A*, *B*, and *C*, shows a representative piece of fundus mucosa from another experiment at higher magnification. In the hematoxylin-stained mother section (Fig. 2, *A*) the distinctly clearer zone of the mucoid neck cells can be easily seen, lying between the surface epithelium, with its configuration of areolae and gastric pits, and the darker stained zone representing the deeper portions of the fundus glands, which contain the chief (pepsinogen) cells. The (dental film) contact autoradiograph (Fig. 2, *B*) unequivocally shows the localization of vitamin B_{12} radioactivity. Quite evidently the mucoid neck cells are completely devoid of radioactivity; it is the deeper zone of the fundus mucosa, corresponding to the basophilic portions of the fundus glands, that contains all of the radioactivity. This exact correspondence to the dark-staining deeper parts of the glands is shown in Fig. 2, *C*, where the autoradiograph is seen projected over the mother section. There cannot, apparently, be much doubt concerning the localization of vitamin B_{12} binding capacity in this section. However, the poor resolution of the dental film contact method does not permit an exact appraisal of the localization on a cellular level.

Figs. 3 and 4, *A* and *B*, show the results of the stripping film autoradiography. Fig. 3 represents the transition between fundus region proper and antrum pylori in a longitudinal section. Apart from the localization in the

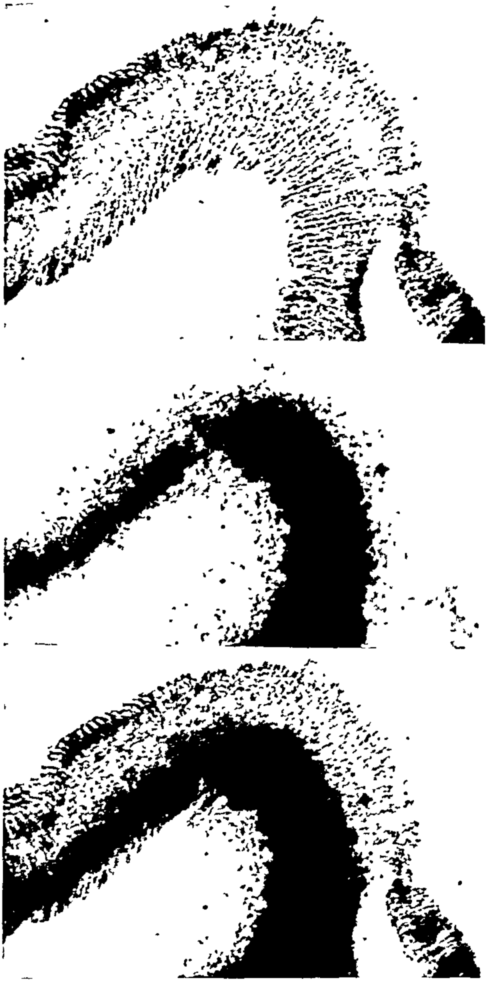

Fig 2—Section of rat stomach, fundus region; incubated with $Co^{57}B_{12}$. *A*, Mother section stained with hematoxylin. *B*, Dental film contact autoradiograph; exposure 3 days. *C*, *B* projected over *A*. Vitamin B_{12} binding is found in deepest portions of fundus glands.

deeper portions of the fundus glands, the abrupt ending of vitamin B_1 binding on the transition can be seen. In Fig. 4, *A*, an unstained section of th fundus with its overlying stripping film is pictured in phase-contrast. Th blackening of the stripping film has the same zonal localization as in the denta film contact autoradiograph. But, in addition, it can be observed here tha only the chief cells are responsible for the blackening of the stripping filr emulsion. The oxyntic cells, rendered visible by phase-contrast, leave unblackened spots in the stripping film. To facilitate appraisal of the autc radiographic picture, the same is reproduced without phase-contrast in ordinary transmitted light in Fig. 4, *B*.

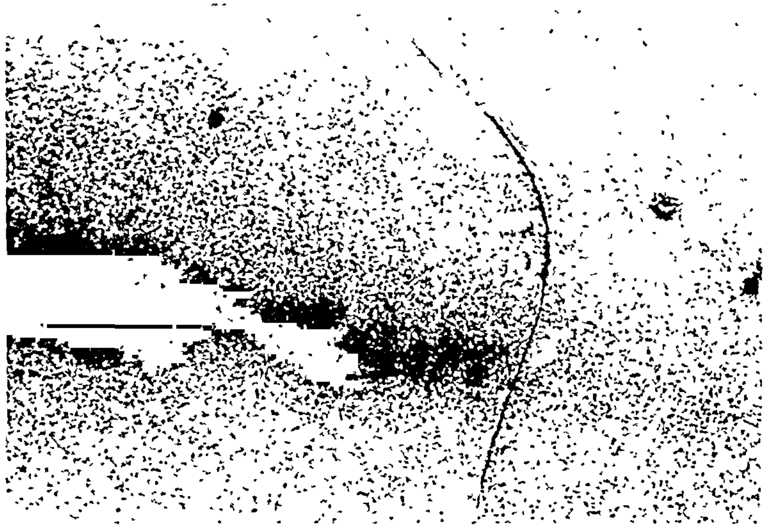

Fig. 3.—Section of rat stomach, transition of fundus region (left) to antrum pylor (right); incubated with $Co^{56,57}B_{12}$ and covered with Kodak AR 50 stripping film; exposure 1 hours. Vitamin B_{12} binding in fundus region, ending abruptly at transition to antrum pylori

The ultimate conclusion, thus, is that under the experimental condition used here localization of vitamin B_{12} binding and thus presumably of intrinsi factor is exclusively in the chief cells of the fundus glands. Neither the mucoi neck cells nor the oxyntic cells did exhibit any vitamin B_{12} binding. Vitami B_{12} binding was equally absent from the mucous surface epithelium of areola and gastric pits in fundus and pyloric regions, and in the mucous glands o the pyloric region. The cardiac region and duodenum also were entirel devoid of vitamin B_{12} binding capacity.

Control Experiments.—The rather unexpected outcome of the autoradiographs induced us to perform several control experiments.

First, a number of paraffin sections from gastric and duodenal wall material fixed in Bouin's solution were stained with the two Bodian-Protargol methods modified according to Dawson[13] for argentaffine cells. Clear pictures were obtained showing these cells scattered singly along the entire length o the gastric glands, both in the fundus and pyloric regions. They were present in even greater numbers in the duodenal mucosa. Neither the regional distribution nor the scattered distribution in the mucosa did show any correspondence whatsoever with the vitamin B_{12} localization observed in the autoradiographs.

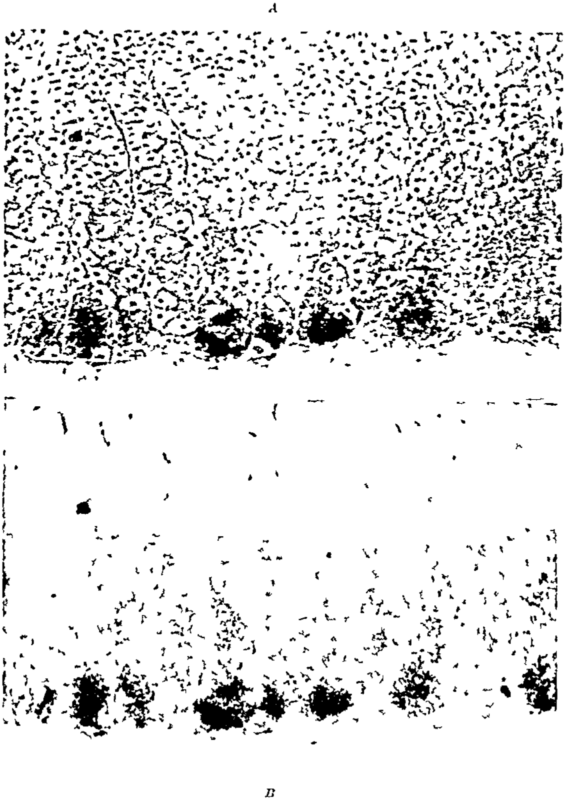

Fig. 4.—Section of rat stomach fundus region incubated with Co^{60}B$_{12}$ and covered with Kodak AR 10 stripping film exposure 24 days. *A* Phase contrast photomicrograph. *B* *Photomicrograph in transmitted light. Vitamin B_{12} binding in chief (pepsinogen) cells of* fundus glands.

Second, a number of experiments were performed to ascertain the presence of biologically active intrinsic factor in the sectioned material at the moment of incubation with vitamin B_{12}. Fundus wall was fixed in cold, saturated ammonium sulfate, homogenized in a Buhler blender at 4° C and the homogenate dialyzed against cold saline. After high-speed centrifugation, the supernatant was tested in totally gastrectomized rats, as described above, and proved to have intrinsic factor activity only slightly less or equal to that of native gastric wall homogenates.

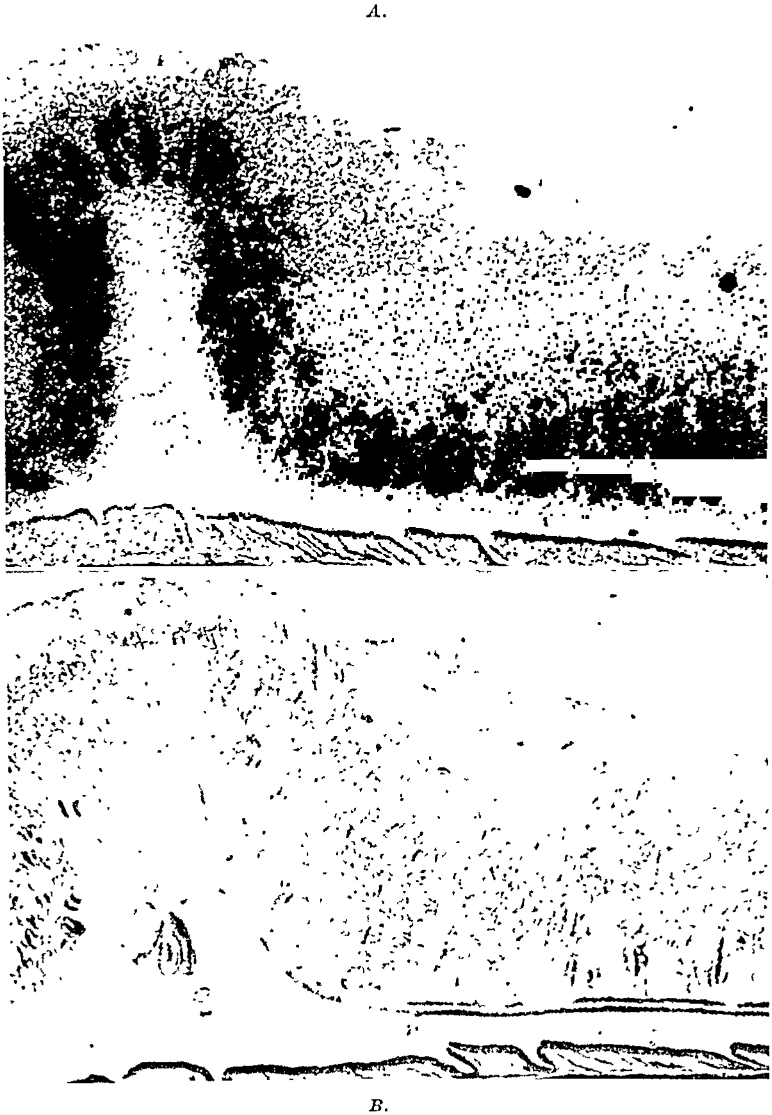

Fig. 5.—Consecutive sections of rat stomach, fundus region. *A*, Incubated with $Co^{56}, {}^{57}B_{12}$ (control section). *B*, Heated to 100° C. for 3 hours and incubated with $Co^{56}, {}^{57}B_{12}$. Both sections covered with Kodak AR 50 stripping film; exposure 24 hours. Vitamin B_{12} binding destroyed by heating.

Third, an experiment was undertaken in which rat pancreatic material was given the complete autoradiographic procedure parallel to the gastric material from the same animal. No vitamin B_{12} binding was seen in pancreatic cells This result excludes the possibility that nucleoprotein material, which is present in comparable quantity and distribution in the chief cells of the fundus glands and in the exocrine pancreatic cells, as such might be responsible for vitamin B_{12} binding in the fundus chief cells

Last, control sections of the fundus region were heated to 100° C in saturated ammonium sulfate for 3 hours, and otherwise treated parallel to the experimental sections Fig 5, *A* and *B*, representing autoradiographs from two consecutive sections of fundus mucosa, clearly shows that heating completely destroyed the vitamin B_{12} binding capacity The resulting autoradiograph (Fig 5, *B*) is entirely negative, whereas the control section presents the usual autographic pattern

DISCUSSION

The biologic part of the present investigation, i e, the experiments with partial gastrectomies and the substitution experiments with stomach wall extracts, has shown beyond any reasonable doubt that in the rat the fundus region of the stomach is the site of production of Castle's intrinsic factor The substitution experiments with the extracts of fundus wall and pyloric regions, if taken by themselves, may not seem entirely convincing in view of their small numbers All data taken together, however, show a striking mutual confirmation which clearly increases their significance

The autoradiographs presented a well defined picture of the localization of vitamin B_{12} binding in the rat gastric mucosa Regarding the regional localization of intrinsic factor, the autoradiographs clearly support the biologic experiments a selective vitamin B_{12} binding was found in the fundus region At a cellular level the localization of vitamin B_{12} binding was unequivocally in the chief (pepsinogen) cells of the fundus glands

The autoradiographs gave no indication whatsoever that the mucoid neck cells play a part in the production of the intrinsic factor Our results clearly suggest that the chief cells are responsible for the elaboration of the intrinsic factor in the rat

This interpretation, however, needs a critical analysis First it should be envisaged that the observed cellular localization might represent a diffusion artefact, as is often seen in histochemistry This possibility can safely be ruled out because intrinsic factor is precipitated by saturated ammonium sulfate, the fixative used Second, it is obvious that the autoradiographic technique used will only demonstrate vitamin B_{12} binding and not intrinsic factor activity itself This is an essential point, because quite a number of unrelated substances have some degree of vitamin B_{12} binding capacity, although most authors agree that intrinsic factor is the most active among them There is, however, good evidence that, under the conditions of our experiments vitamin B_{12} binding represents the presence of intrinsic factor A first argument is that our procedure, with saturated ammonium sulfate as a fixative, was shown not to destroy intrinsic factor activity in the pertinent control experiment This means that, for all we know, intrinsic factor must have been responsible for most, if not all, of the vitamin B_{12} binding observed A second argument comes from a control experiment; upon heating to 100° C., a temperature known to destroy intrinsic factor, the sections completely failed to take up vitamin B_{12}.

We believe, therefore, that our autoradiographs are fairly conclusive with regard to the localization of intrinsic factor in the chief cells of the fundus glands, and, consequently, that these cells should be considered as the presumable site of production of intrinsic factor in the rat.

Cytologically, the chief cells of the fundus gland have the typical characteristics of protein-synthesizing cells, whereas any sign suggesting the production of a mucoid or polysaccharide is lacking. This suggests that the active principle of the intrinsic factor might be a protein or a polypeptide rather than a mucoid or a polysaccharide. This hypothesis is in argreement with the observation of Cresseri,[8] cited above, that the vitamin B_{12} binding substance in electropherograms of glandular mucoprotein (which presumably contains intrinsic factor) contained a protein moiety as shown by its bromphenol staining.

However, it should be mentioned that experiments with hog gastric mucosa, now in progress, have shown that conditions in this animal are more complicated than in the rat. This holds for the biologic and perhaps also for the chemical properties of intrinsic factor as well as for the presence of substances exhibiting nonspecific vitamin B_{12} binding. Species differences like these might be responsible for much of the confusion in the intrinsic factor problem.

SUMMARY

Two series of experiments were performed with the purpose of determining the site of production of Castle's intrinsic factor in the rat.

The intestinal absorption of radioactive vitamin B_{12} was studied in rats in which different parts of the stomach had been resected. The data obtained in these animals indicated that the fundus is essential for normal vitamin B_{12} absorption and, therefore, can be considered responsible for the production of the intrinsic factor. This was confirmed by testing extracts of the different parts of the rat stomach for intrinsic factor activity in rats in which total gastrectomy had been performed.

In the second part of this study a number of histologic techniques were applied in an attempt to determine the type of cell responsible for the secretion of the intrinsic factor. The most significant information was obtained by autoradiography of sections incubated with radioactive vitamin B_{12}. Selective uptake of vitamin B_{12} occurred in the chief (pepsinogen) cells of the fundus glands. Presumably this cell type elaborates Castle's intrinsic factor in the rat.

We wish to express our sincerest thanks to N. V. Philips-Roxane, Weesp-Holland, for the generous gifts of radioactive vitamin B_{12}. Their willingness to take up the preparation of this valuable and costly material provided us with the main tool for the present investigation.

The histologic work, including autoradiography, was done by Miss E Binkhorst (Department of Histology) Microphotography was by Dr J Wachters (University Photographic Department) Dr J Abels assisted in some of the biologic control experiments

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EVALUATION OF A METHOD OF DIRECT LEFT HEART PUNCTURE

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PERCUTANEOUS needle puncture of the left ventricle has been advocated as a relatively safe method for pressure measurement and cardioangiographic study. It has been done for the most part in adults, and is performed only occasionally in children.[1-6] While it is often easy to pass a venous catheter into the left side of the heart of infants through the probe-patent foramen ovale, when access to the left side is most urgently needed it may be impossible to gain. *It seemed desirable to develop a method which would have special attributes* for work with infants and small children. These would be: (1) a simple aiming technique which would assure puncture of the intended chamber despite absolute and relative variation in size of structures from patient to patient; (2) minimal effect of practice upon the likelihood of success in the event that the procedure was utilized only occasionally; and (3) use of a tube of sufficiently large bore to permit satisfactory contrast injections for cardioangiography. It is believed that the method to be described below promises to fulfill these criteria in patients. It has the added advantage of permitting catheterization from the ventricle through the aortic valve and retrograde through the mitral valve. This paper reports the trial of the method in a series of 12 rabbits. This experimental animal was selected because of the smallness of the heart, thus pitting the method against a maximal handicap. It was also tried in 6 mongrel dogs to study its use in larger hearts. Finally, left ventricular puncture using this approach was performed successfully and apparently harmlessly in a 3-month-old infant with severe congenital heart disease.

MATERIALS AND METHODS

A stylet (Fig. 1, *A*) made of a 19 gauge steel piano wire, 16 cm. in length was sharpened at one end to a needle point, and bent at a right angle 1.5 cm. from the other end. A piece of polyethylene† or tetrafluorethylene‡ tubing (Fig. 1, *G*) through which the wire could be threaded snugly was cut. A piece 1.5 cm. long (Fig. 1, *D*) was cut from a 16 gauge hollow needle§ and inserted through one end of the tubing. This necessitated slight stretching of the plastic, the internal diameter of which approximately equaled that

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†Intramedic internal diameter, 0.047 inch, outside diameter, 0.067 inch. Clay-Adams, Inc., New York, N. Y.

‡Teflon (Dupont) thin-walled spaghetti tubing No SP-600, AWG 18, internal diameter, 0.042 inch; outside diameter, 0.066 inch. Sparta Manufacturing Company, Dover, Ohio.

§Standard needle, Becton, Dickinson & Company, Rutherford, N. J.

of the 16 gauge needle To this segment of needle, a removable adapter* (Fig 1, B, C, F, and F) was fitted The stylet was threaded through the adapter and tubing so that only its sharpened end protruded A piece of No 7 cardiac catheter (Fig 1, H) 3 mm in length was then passed over the tubing to serve as a marker

For use in infants and small children, the stylet was replaced with a 19 gauge hypodermic needle, 25 cm in length and joined to the hub of a 16 gauge needle A tetrafluorethylene tubing was used, the end of which was cut and shaped to fit the point of the needle

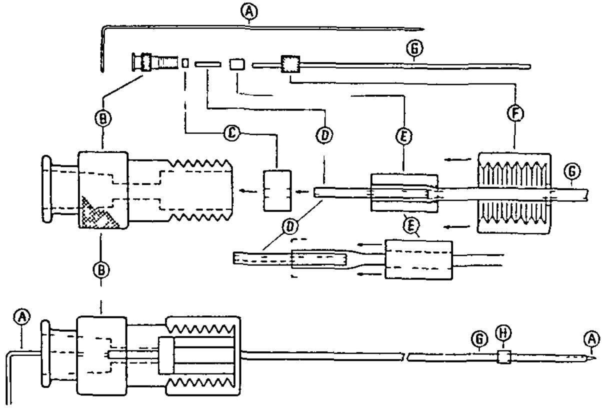

Fig 1—Stylet tubing instrument for percutaneous ventricular puncture A = stylet B = adapter body C = rubber gasket D = piece of 16 gauge needle E = steel gland F = steel gland nut G = polyethylene or Teflon tubing H = depth marker

1 *Animal Experiments*—Twelve rabbits with an average weight of 3 Kg were obtained

An unanesthetized rabbit was strapped by its legs to a board The anterior chest was shaved and cleansed with Zephiran solution 1 1,000 ECG needle electrodes were placed subcutaneously in the legs A skin stab wound 2 mm in length was made over the left paraxiphoid area The stylet and polyethylene tubing (Fig 1, A and G) were inserted through the incision and subcutaneous tissues, and into the thoracic cavity Under fluoroscopic control with an image intensifier, the tip was directed toward the apex of the heart at a 35 degree angle and very slightly to the right As soon as pulsations were felt, the stylet tubing was quickly thrust forward into the heart, the marker (Fig 1, H) being placed at an estimated proper distance of insertion so as to serve as a safety guide When the ventricular cavity was entered, the stylet was removed and the tubing was advanced slightly A 3 way stopcock was attached to the adapter (Fig 1, B) Ventricular pressures were recorded with a Hathaway impedance gauge The tubing could be advanced and retracted easily in various directions It was filled with contrast medium† which rendered it visible, then the 3 way stopcock was rotated to connect the tubing with the pressure gauge, without losing the contrast medium This permitted the tube to be seen and the pressure in the chamber to be constantly observed on the oscilloscope The tubing was passed into the outflow artery, and into the atrium It was occasionally flushed with 1 to

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*U S Catheter & Instrument Company Glens Falls N Y

†Hypaque M 90 per cent Winthrop Laboratories New York N Y

2 ml. of normal saline solution, containing 1 mg. of heparin in 500 ml. of fluid. After pressure measurements were taken, the tubing was removed. Light pressure and adhesive tape were applied over the wound. The rabbit was freed and returned to its cage.

Gross postmortem examination was performed in rabbits that died shortly after the procedure. Histologic studies were done in those which were killed with pentobarbital sodium given intravenously 8 or 9 days, 16 days, 36 days, and 43 days after the ventricular puncture.

The procedure was tried in 5 anesthetized mongrel dogs, weighing 11.5 to 13.5 kilograms. The stylet and tubing were inserted through the fourth left intercostal space anteriorly. Four of these were used in other experimental studies, and were sacrificed after being subjected to ventricular puncture. One was kept alive and sacrificed after 3 weeks.

The heart was exposed in an anesthetized dog weighing 24 kilograms, the pericardium being left intact.. The stylet and tubing were thrust successively through the anterior wall of the left and right ventricles, then into the left and right atria. The pericardium was opened, and the 4 chambers were again punctured. The main left anterior descending coronary artery and 2 of its terminal branches were also punctured with the stylet.

2. *Clinical Trial in One Case.*—S. P., a 3-month-old infant weighing 11 pounds and 7 ounces, had repeated respiratory tract infection and pulmonary congestion, cardiomegaly, and enlarged liver. Right heart catheterization and selective cineangiocardiography were inadequate in arriving at a diagnosis because of inability to localize the shunt by blood oxygen saturation studies, and poor visualization of the left side of the heart and aorta due to insufficient concentration of contrast medium. The possible diagnoses entertained were truncus arteriosus; common atrioventricular canal; and ventricular septal defect, alone or combined with an atrial septal defect, patent ductus arteriosus, or coarctation of the aorta. Left heart studies were done to arrive at a more definitive diagnosis. The patient was anesthetized with intramuscular meperidine hydrochloride and secobarbital sodium. The fifth left intercostal space at the midclavicular line was infiltrated with procaine hydrochloride; a stab wound of the skin 2 mm. long was made. The instrument was thrust in through the apex of the heart. A 1 ml. syringe containing 70 per cent Urokon was attached to the stylet needle. When the point was shown to be in the ventricle by aspiration of blood into the syringe, contrast medium was injected as the needle was retracted and the tubing slightly advanced.

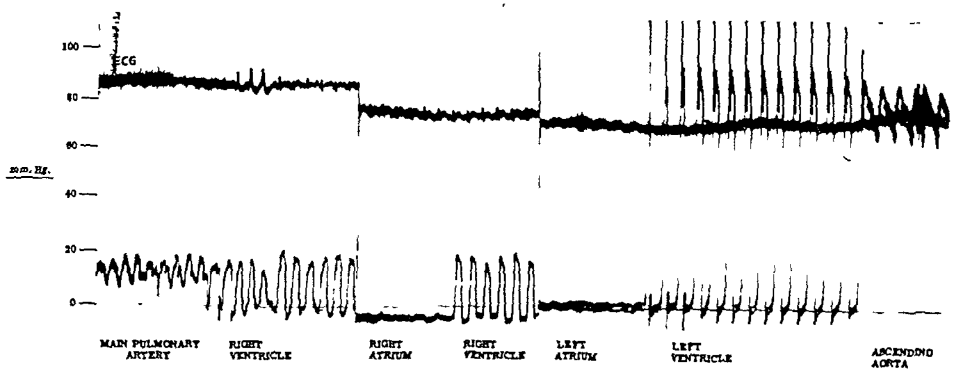

Fig. 2.—Pressure tracings in both ventricles through the atrioventricular and semilunar valves in rabbits. Note systolic overshoot artefact in left ventricle.

RESULTS

The rabbit's heart was not fixed in the mediastinum. It was easily displaced by just placing the stylet tubing in the thoracic cavity and feeling for ventricular contractions. Once the ventricular cavity was entered, the pulsations imparted by the heart to the stylet diminished or disappeared. *If the tubing was in the left ventricle, bright red blood spurted from it. Dark blood* flowed slowly from the tubing if the right ventricle had been entered instead.

TABLE I. PHYSIOLOGIC DATA*

RABBIT NO.	COURSE OF TUBING	PRESSURES	ECG
1	LV AA LV	AA = 63/49 LV = 67/-6	CR = 240/min (during puncture) CR = 120/min *(end of procedure)*
2	LV AA	LV = 66/-1 AA = 65/51	CR = 180/min
3	RV MPA RV	RV = 16/-5 MPA = 18/4	CR = 260/min PVC, 1 to 3, with tubing in RVO
4	LV AA LV LA LV	AA = 106/85 LV = 104/-2 LA = 0.5 mm	CR = 240/min Slight S-T elevation with tubing in LV, none in AA or LA
5	RV MPA RV	MPA = 8/4 RV = 10/-6	CR = 200/min Rare PVC with tubing in RVO
6	RV RA RV	RV = 17/-6 RA = -9 mm	CR = 240/min
7	LV AA LV LA LV	LV = 97/-0.8 AA = 95/82 LA = 1.3 mm	CR = 320/min
8	LV LA LV AA	AA = 116/96 LV = 112/-2 LA = -6.7 mm LV = 109/-2	CR = 200/min
9	LV AA LV LA	AA = 94/85 LV = 103/0.6 LA = -6.4 mm LV = 107/-5	CR = 300/min S-T elevation
10	RV RA	RV = 15/-2 RA = -6 mm	CR = 220/min
11	LV AA LV LA	LV = 86/-1 AA = 89/70 LA = -2 mm	CR = 200/min
12	RV IV septum LV AA LV LA LV RV RA RV	LA = -7 mm LV = 84/-0.3 AA = 79/63 RA = -7 mm RV = 14/-2	CR = 260/min S-T elevation Few PVC with tubing in RVO

*Key to Abbreviations: LV = left ventricle; AA = ascending aorta; LA = left atrium; RV = right ventricle; MPA = main pulmonary artery; RA = right atrium; RVO = right ventricular outflow; IV = interventricular; CR = cardiac rate; PVC = premature ventricular contractions.

The tubing was passed into the main pulmonary artery or aorta, and into the left or right atrium, depending on which ventricle was entered. Good pressure curves were obtained (Fig. 2 and Table I). In Rabbit 12, the stylet pierced the right ventricle and went through the interventricular septum into the left ventricle. Pressure tracings were obtained from both ventricles and atria and the ascending aorta. Incidence and amount of bleeding were increased considerably when more than one attempted puncture was made, and bleeding was

2 ml. of normal saline solution, containing 1 mg. of heparin in 500 ml. of fluid. After pressure measurements were taken, the tubing was removed. Light pressure and adhesive tape were applied over the wound. The rabbit was freed and returned to its cage.

Gross postmortem examination was performed in rabbits that died shortly after the procedure. Histologic studies were done in those which were killed with pentobarbital sodium given intravenously 8 or 9 days, 16 days, 36 days, and 43 days after the ventricular puncture.

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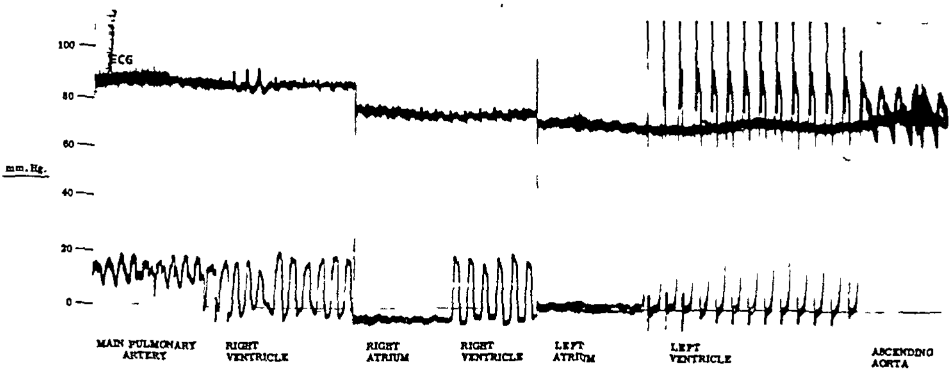

Fig. 2.—Pressure tracings in both ventricles through the atrioventricular and semilunar valves in rabbits. Note systolic overshoot artefact in left ventricle.

RESULTS

The rabbit's heart was not fixed in the mediastinum. It was easily displaced by just placing the stylet tubing in the thoracic cavity and feeling for

When multiple punctures were done through the different chambers and coronary vessels in the dog's exposed heart, slight bleeding occurred when the pericardial vessels were pierced There was no bleeding from the wounds in the ventricular wall Blood oozed from the atrial puncture wounds in negligible amounts when the pericardium was intact With the pericardium open, the second atrial punctures did not cease bleeding until direct pressure was applied Blood spurted when the left anterior descending coronary artery was pierced, and oozed from the corresponding vein when the latter was punctured in turn There was minimal bleeding when the terminal coronary artery branches were subjected to puncture

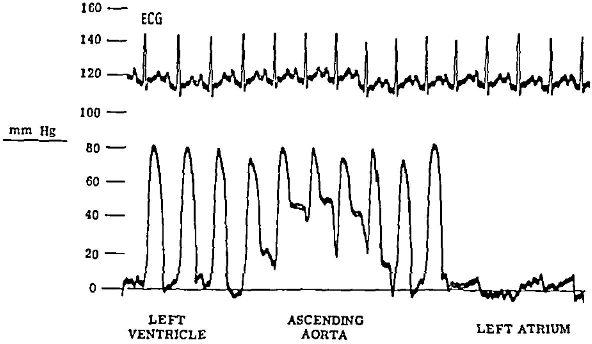

Fig 3—Pressure recording taken during manipulation of catheter through the precordium of a 3 month old infant The low aortic diastolic pressure in some of the complexes indicates aortic insufficiency The variability of this finding suggests that it is an artefact due to pressure of the catheter on the valve

In the 3 month old infant, moderate resistance was offered by the subcutaneous tissues and the hypertrophied left ventricular wall The left ventricle was entered without undue arrhythmia The tubing was passed into the aorta and left atrium, and pressure recordings were taken (Fig 3) However, the tip of the tubing slipped back into the pericardial sac before satisfactory injection of contrast medium could be made A second puncture was made with greater ease, and left ventricular cineangiocardiography was performed The study showed a ventricular septal defect and narrowing of the aorta, but no discrete coarctation The child tolerated the procedure well, and was wide awake and fairly active when brought back to the ward Follow up over a 1 month period indicated no evidence of delayed ill effects

DISCUSSION

It is believed that this new method has distinct advantages over needle punctures into the left atrium or left ventricle for measurement of pressures or injection of contrast medium directly, and over the polyethylene tubing threaded through a needle and passed subsequently through the different cardiac chambers. Ordinarily, 15 to 18 gauge needles are used for left heart punctures through the chest wall, and one corresponding to a 22 gauge needle by the suprasternal approach. The stylet or needle used in our studies is of 19 gauge. Since the stylet is threaded through the plastic tubing, and removed after it is in a ventricular chamber, a large bore tubing remains in place. This tubing corresponds in outside diameter to a No. 5 French cardiac catheter. It is about twice the external diameter of the left heart catheterization tubing passed through a 17 gauge transbronchial needle. Its larger lumen lessens the incidence of clotting, and enables one to get better pressure tracings and inject a satisfactory amount of contrast medium rapidly. The ventricular wall can clamp more effectively on the slightly compressible tubing than on a needle, preventing leakage. Being flexible, this tubing produces less fixation of the heart and should be better tolerated than a rigid needle of the same or larger size. Furthermore, the tubing is of sufficient size and rigidity to permit considerable manipulation in achieving passage through the atrioventricular and semilunar valves. Reasonably accurate pressure curves are obtainable through both the polyethylene and Teflon tubes.

The rabbits used for the study were young, and had hearts which weighed about 4 grams, as compared to about 100 grams in dogs weighing 12 kilograms. Considering the size of the hearts and the thickness of the ventricular wall in relation to the size of the puncture wound in the myocardium, there was remarkably little bleeding into the pericardial and thoracic cavities when the first attempt was successful. Possibly a pericardial or internal mammary vessel was punctured in the eleventh animal to account for the blood clot in the inferior mediastinum. In the first rabbit, there was gross evidence that a major coronary artery had been pierced twice. There were only minimal S-T changes observed while the experiment was in progress. Unfortunately, an electrocardiogram was not taken before the rabbit was sacrificed to look for further changes, if any. Presumably, the rabbits had normal myocardia so that the rate of healing was fairly rapid.

In dogs, except for some difficulty in going through the chest wall, the procedure was easier than in rabbits. The diaphragm of the dog is more conical and higher. It was difficult to avoid the left leaf of the diaphragm by the paraxiphoid approach. The stylet and tubing, therefore, were inserted through the fourth left intercostal space. Apparently the wounds produced in the left ventricular wall closed immediately upon withdrawal of the tubing so that bleeding was not observed to come from them. Autopsy studies have been reported on dogs punctured with a 17 gauge needle; the sites of entry were often difficult to identify, and detected only microscopically as focal scarring.[1]

As shown by multiple thrusts into the exposed heart of a dog, it could be that even in our rabbit experiments, bleeding did not always come from the puncture sites of both ventricles, but possibly came from the pericardial vessels or coronary blood supply when these were pierced by the stylet.

For infants and small children, a piece of tetrafluorethylene tubing, 25 cm. in length was thought sufficient to reach from the apex of the heart to the proximal aorta A 19 gauge hypodermic needle was used instead of a wire stylet in order to indicate whether the tip of the instrument was inside the pericardial cavity or in a cardiac chamber before removal of the needle, and also, in order to avoid an embolism, by permitting the introduction of contrast fluid *into the tube as the needle was withdrawn* *Approaching the left ventricle through the apex reduced the possibility of piercing large branches of the coronary arteries* Also, the internal mammary vessels were avoided However, the pleural space was probably traversed The procedure should be tolerated by the ventricular myocardium in young children whose coronary circulation is normal Since the scar is weakest during the second week after the puncture, restriction of activity may be desirable

Note While the experiments were in progress, Seldinger[7] described a remarkably similar stylet catheter for splenic puncture in lienoportal venography and percutaneous transhepatic cholangiography Earlier he had applied a similar instrument to the puncture of arteries and veins[8, 9]

SUMMARY

A new method for percutaneous left ventricular puncture is described A 19 gauge stylet threaded through a polyethylene or Teflon tubing was directed into the left ventricle through the left paraxiphoid angle in rabbits, and through the fourth left intercostal space anteriorly in dogs The stylet or needle was then withdrawn, leaving the tubing in place in the ventricular cavity The tubing was passed into the aorta and left atrium, and pressure tracings were taken In rabbits, bleeding was negligible when the first attempt was successful, but its incidence and amount were increased greatly when more than one attempt was made Microscopically, there was early scar formation and intact visceral pericardium and endocardium In dogs the left ventricle was easier to puncture and no bleeding occurred Direct visualization of multiple punctures of a dog's heart and pericardium showed that ventricular and small branch coronary artery bleeding were insignificant, while the pericardial vessels and atria might bleed enough to require pericardial tapping As the procedure seemed reasonably safe to apply in infants and small children, it was performed in a 3 month old infant severely ill with an incompletely diagnosed congenital anomaly of the heart The solid stylet was replaced by a hollow needle stylet The stylet tubing was introduced at the apex The left ventricle was entered, the left atrium and aorta were catheterized, and satisfactory cardioangiography was performed, without ill effects

We wish to acknowledge the assistance with the histology provided by Dr Serafin Juliano, ICA Fellow in Pathology, Department of Pathology, Indiana University School of Medicine Mr Clyde Foster gave valuable technical assistance Dr Harold King of the Department of Surgery originally suggested the idea of a stylet catheter to us

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LABORATORY METHODS

QUANTITATIVE DETERMINATION OF FREE LYSINE IN URINE BY TWO DIMENSIONAL PAPER CHROMATOGRAPHY

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Melbourne, Australia

IN THEIR earlier surveys of amino acid excretion in various disorders Dent and associates[1] [2] failed to resolve lysine and ornithine and lysine and arginine Since preliminary observations of amino acid excretion during pregnancy indicated that fluctuations of lysine concentration appeared greater than that of any of the other amino acids, conditions were sought under which this amino acid could be unequivocally determined

The present modification was designed to give a quantitative determination of lysine by the optical scanning method using an "Eel scanner* This was achieved by (1) keeping the specimen at pH 2, enabling a standard to be run and measured on the same paper side by side with the unknown under, as nearly as possible, the same conditions (at this pH a compact spot of lysine dihydrochloride was obtained), and (2) enlarging the area of the applied sample contrary to the generally accepted practice, to give a lysine spot large enough to be measured in the Eel scanner

The use of the Dent apparatus allowed as many as 10 determinations to be carried out together

MATERIALS AND METHODS

Apparatus—The two dimensional capillary ascent technique was used with stainless steel apparatus, as described by Datta and associates[3] which allows minimum handling of the sheets

Paper—The paper used was Whatman No 1[4] Each sheet measured 12 by 12 inches

Solvents—Eighty per cent phenol in water was used as the first solvent Since the method depends on a particular form of pH control, ammonia was not necessary This also minimizes background color which has been shown to be responsible for high and variable blanks[6] [14] A R grade B D H † phenol was found to be satisfactory without further purification, and the solution could be reused[7] as many as 4 times until a pink color developed

The second solvent was a 65 per cent solution of a mixture of 2 4 and 2 5 lutidine (A R grade B D H) A number of water miscible solvents,[8] 60 per cent acetone, 75 per cent ethanol,

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*Manufactured by Evans Electroselenium Ltd Harlow Essex England

† Analytical reagent grade British Drug Houses Ltd England

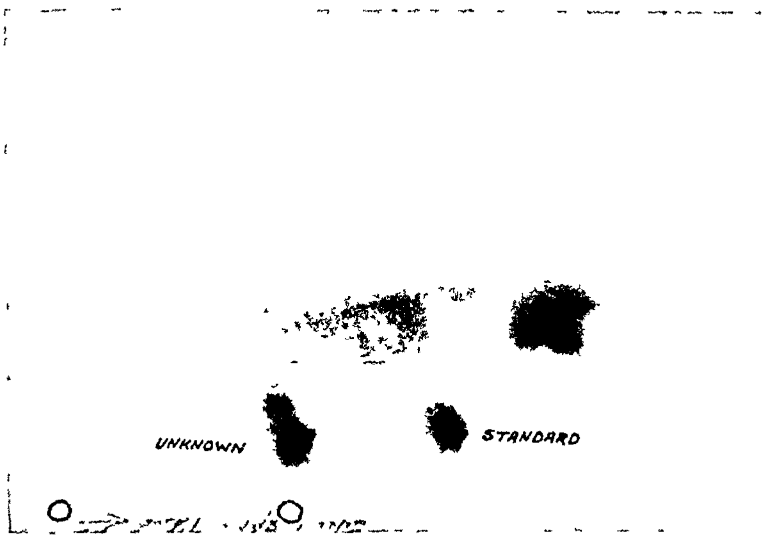

Fig. 1.—Illustrated are the final positions of lysine in the urine specimen (pH 2.2 the standard lysine (pH 1.4) after completion of the two dimensional run. The strip fit the scanner can be seen.

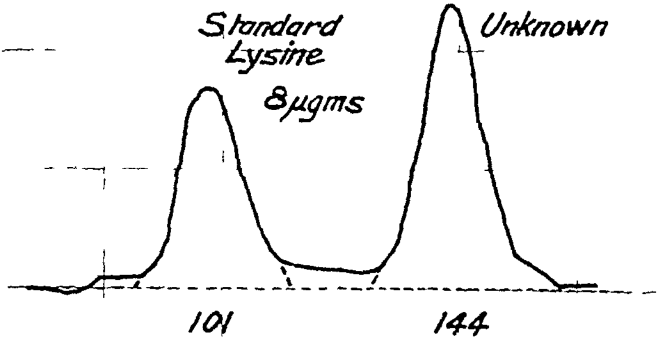

Fig. 2.—The optical density curves of the lysine spots shown in Fig. 1.

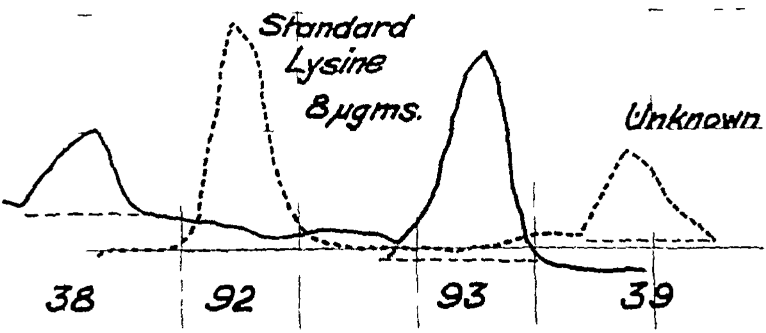

Fig. 3.—Scanner curves show result of reversing strip in holder to obtain base line

n-butanol-acetic acid-water mixture (40:10:50), was tested, but they were found to be unsuitable.

Development of Spots.—One tenth per cent ninhydrin in 95 per cent ethanol was sprayed on the sheets, which were allowed to dry overnight at room temperature to obtain maximum development of the colors.[9]

Specimens.—Twenty-four hour urine specimens containing 50 ml. 1.5N HCl as preservative were each made up to 2 liters. To ensure that collection of the specimens was complete, creatinine was determined.

Desalting.—This[10, 11] was considered unnecessary as in the few cases where a "salt effect" was noticed the lysine spot was not affected.

Technique.—In order to obtain spots of a size suitable for measurement in the Eel scanner, volumes of urine somewhat greater than generally recommended were used.

One hundred microliters of the diluted specimen (pH 2) were placed at the lower left-hand corner of the paper, 1½ inches from the lower edge and 1 inch from the left-hand side. An 0.1 ml. micropipette was used to add the specimen a drop at a time. Each drop was dried immediately by means of an electric hair dryer to ensure the diameter of the applied spot to be about 1.5 cm.

It was found that this volume of urine gave approximately 2 to 10 μg of lysine, a conveniently measureable quantity. For the minimum quantity of lysine which gives visible colors with ninhydrin, varying figures are given dependent on the conditions.[12-14]

Eight microliters (1 μg per microliter) of standard lysine solution of pH 2 was delivered by means of a calibrated red blood cell micropipette at a point 3 inches to the right of the origin and allowed to spread to the same area as the unknown. Duplicate samples were treated with H_2O_2[12, 15] and lysine appeared to be unaffected.

The temperature of the chromatography room varied only from 20 to 22° C. The sheets were allowed to equilibrate overnight for at least 18 hours.[16] After the phenol run the sheets were dried at room temperature for about 3 hours and finally were transferred to a thermostatically controlled cupboard equipped with a fan, and dried at 35 to 40° C. for an additional 6 hours. By this time no smell of phenol remained.

The phenol is not easily removed at room temperature. On the other hand, if dried at high temperature losses of amino acids occur.[5, 14, 17, 18] Under our conditions the phenol is completely removed at a temperature which should result in a minimum loss of amino acids.

Since the standard and unknown lysine were run on the same sheet and their concentrations were of the same order of magnitude, any slight loss should not affect the final result which is a relative figure only.

After equilibration for 2 to 3 hours the lutidine solution was run for 24 hours.

The use of the basic solvent first[10] would reduce considerably the total running time of the chromatogram, since drying of the sheets after each run would be quicker. However, Block[19] found that under these conditions the color intensities were less than when phenol is used first.

After development with ninhydrin the piece of sheet containing the unknown and standard lysine spots was cut out to fit an Eel scanner (Fig. 1). The optical density was read on a calibrated microammeter, and the reading was plotted directly to give a graph of the concentrations. The areas under the two peaks were measured by counting the squares on the graph paper and the unknown calculated as milligrams per 24 hours (Fig. 2).

The Eel scanner is normally used to measure proteins after separation by electrophoresis. It is a simplification of an instrument such as that described by Grassmann and Hannig.[24]

The other constituents of the urine affect the standard less than they do the unknown. Other amino acids have been in contact with the paper between the unknown and the standard during the phenol run, and may have left a slight residue detectable by the scanner. In some cases the graph did not return to the original zero position. In an attempt to cancel out this effect, after scanning in the direction standard to unknown, the strip was reversed in the holder to measure in the opposite direction (Fig. 3).

The length of the slit of the scanner is 3½ cm. The originally applied spots of ur and of standard of diameter 1½ cm. gave lysine spots each of diameter 2½ cm. This lef blank margin of ½ cm. on the paper both above and below the spot. The length of the slit fixed; therefore, we attempted to remove the effect of the blank margins by placing strips black paper on the glass plates but the curves ran off the graph paper. It was found m satisfactory to leave the blank margins provided care was taken to keep them the same wi for both unknown and standard. The results for two pairs of lysine spots of different c centrations, each pair run at different times, show this effect (Table I).

TABLE I

	(A) LARGER BLANK MARGINS		(B) SMALLER BLANK MARGI	
Lysine in μg	5.6	11.2	1.6	4
Area in $\frac{1}{10}$ inch squares	48	90	42	94

After replacing the cut-out piece of paper with transparent tape the whole sheet then photographed in transmitted light both in color and in black and white.

Finally, in anticipation of fading, the individual spots on the sheets were outlined pencil and a description of the color and relative strengths was written beside each spot.

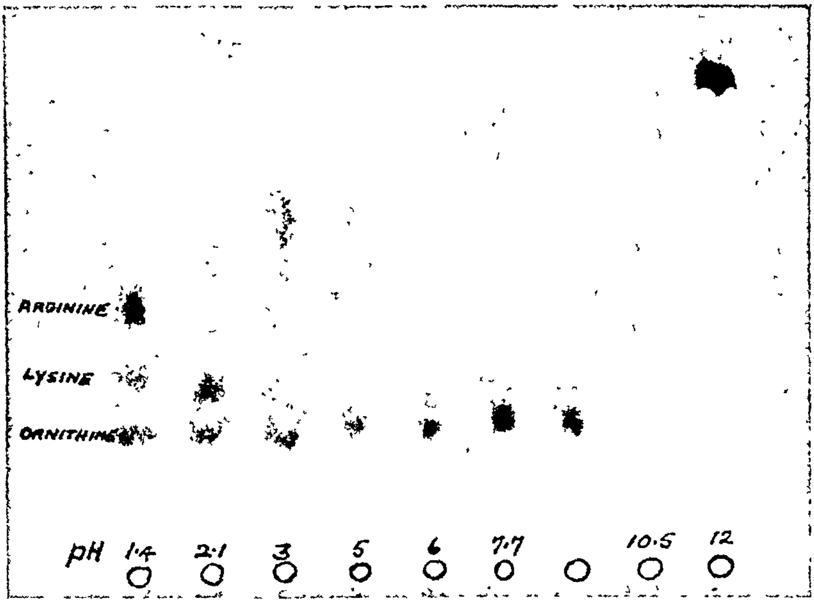

Fig. 4.—Four microgram mixtures of ornthine, lysine, and arginine at different pH's af phenol run.

RESULTS AND DISCUSSION

Arginine can occur in urine, and Stein[20] found large excretions of ornithi as well as lysine and arginine in the urine of 5 patients with cystinuria.

At pH 1 to 2 in a phenol-water system lysine gives a single compact spot the dihydrochloride, while between pH 2 and 10 it gives long streaks due to d sociation of the carboxyl group.[21, 22] At pH 11 to 12 a single spot again occu since the two amino groups have lost their protons, this represents the ani Arginine and ornithine behave in a similar fashion.

With a mixture of arginine, lysine, and ornithine, discrete spots appear at and below pH 2, but at pH 11 to 12 the 3 coincide at Rf 0.75 This behavior is shown in Fig. 4.

To ensure that the unknown spot being studied is, in fact, lysine, identification was established by adding known amounts of lysine, arginine, and ornithine to the urine sample for the two-dimensional run. Only the added lysine coincided with the unknown spot (Figs 5 and 6).

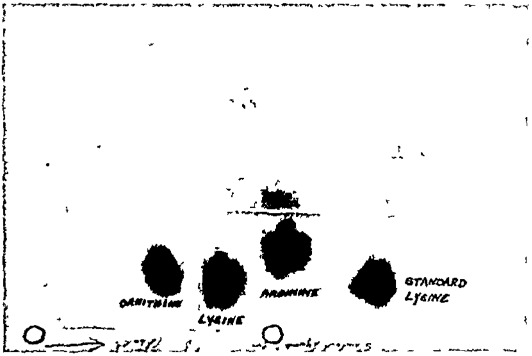

Fig. 5—Chromatogram of urine specimen (pH 2) shows quantitative measurement of lysine in presence of ornithine and arginine Eight micrograms each of arginine, lysine and ornithine has been added to the specimen which originally contained no detectable lysine

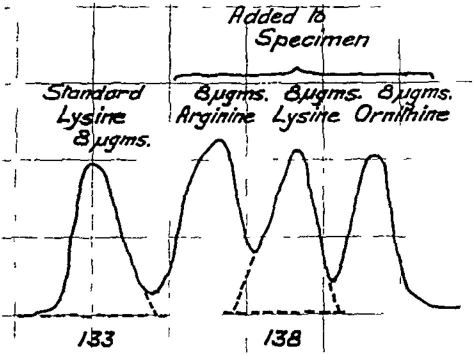

Fig 6—Scanner curves of spots shown in Fig 5

Landua and associates[22] suggest pH 10 as the most suitable condition for the separation of lysine Our results show that this is not desirable when arginine or ornithine is present.

A pH of 1 to 2 is not necessarily the most suitable pH for other amino acids which occur in urine. At this pH some give rather large and, in some cases, diffuse spots, although preliminary experiments have shown that taurine, cystine, alanine, and glutamine may also be determined on the same paper as the other 3. Therefore, if amino acids other than these are to be determined, some other pH will be necessary or, alternatively, other solvents must be used.

Stein and Carey[23] have shown that the concentration of lysine in urine increases after acid hydrolysis. Our conditions are very mild compared with those necessary for hydrolysis, and no change has been observed after 8 months' storage in a refrigerator.

It cannot be stressed too much that in all chromatographic work it is imperative to know exactly the conditions under which the experiments have been carried out before any interpretation of a chromatogram is attempted. For example under the conditions of our experiments (pH 1 to 2) glutamic acid appears as a horizontally streaked spot above lysine (Fig. 5). Under alkaline conditions Dent and Walshe[2] have shown glutamic acid as a compact spot appearing in approximately the same position as lysine does under our conditions.

SUMMARY

A two-dimensional chromatographic method for the determination of lysine in urine has been described. A standard is run on the same paper. By adjustment of the pH of the samples and of the area over which they are allowed to spread when applied, spots of one lysine species are obtained which can be compared by means of an Eel scanner.

I wish to thank Miss M. Johnson (Pathology Department, Royal Women's Hospital) for the photographic work, and Mr. J. W. Legge (Biochemistry Department, University of Melbourne) for his helpful criticism during the writing of this paper.

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DIFFERENTIAL ULTRAVIOLET SPECTROPHOTOMETRIC DETERMINATION OF SERUM SALICYLATES

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The quantitative determination of salicylates in biologic media has seen useful employment for clinical,[1] forensic,[1] and pharmacologic applications.[3] The determinative techniques have been mainly concerned with the phenolic nature of salicylate either through iron or metal reactions[6, 7, 9] or by coupling with diazotized p-nitroaniline.[5] Some investigation has been carried out on the reducing action of salicylates[8] or on the determination of salicylic acid in the ultraviolet region of the spectrum.[10] The problem of the isolation of salicylic acid for quantitative and qualitative ultraviolet spectrophotometry free from the interferences encountered in biologic material can be simplified by the use of the differential characteristics of the ultraviolet spectra of salicylate at two different pH levels. Previously, it has been shown that the spectrophotometric determination of barbiturates can be carried out by the employment of automatic recording differential spectrophotometry.[12] This has provided a simple, rapid, and accurate ultraviolet spectrophotometric quantitation procedure in which a composite spectrum could be obtained. This spectrum represented the *automatic subtraction of two spectra, both containing the same substance* in the same concentration but at two different pH levels. The conversion of the solution from pH 13 to pH 10.5 resulted in a hypsochromic shift of the spectrum. The subsequent subtraction of one spectrum from the other spectrum showed characteristic differences peculiar to the barbiturate being investigated.[11]

The present investigation describes the use of an automatic ratio recording spectrophotometer for the quantitative determination of the differential spectral characteristics of salicylate at two pH levels. As previously described for barbiturates,[12] it obviates the time and error involved in manually determining spectra which are crude and limited in nature[2]; it does not use blanks since the two aliquots of salicylate blank each other just as if the spectra had been individually recorded against their own blanks; it eliminates subtraction of one spectrum from its mate since the instrumentation technique automatically substracts the two spectra, and it enables one to analyze for salicylate or barbiturate or a mixture of the two as they may be encountered in biologic media. The extension of the procedure to 100 μl samples for salicylate determination

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is also described. The automatic ratio recording spectrophotometer is not necessary to the procedure since a manual spectrophotometer can also be used. However, automatic instrumentation effects a great saving in the time and effort involved in the technique to be described.

MATERIALS AND METHODS

Reagents.—

1. Chloroform, spectranalyzed or redistilled
2. Sodium hydroxide, 0.45 N
3. Ammonium chloride, 16 and 20 per cent
4. Hydrochloric acid, 6 N

Equipment.—

1. Beckman DK 2 automatic ratio recording spectrophotometer
2. Silica cuvettes, macro and micro, 1 cm light paths

Instrumentation.—Zero the instrument, utilizing the hydrogen lamp source and the photomultiplier tube at position IX, using the following specifications:

Wave length	400 mμ
Approximate slit width	0.04 mm
Sensitivity	100
Time	2 minutes
Time constant	0.2
Initial range	0 to 100
Scale, initial	per cent T
Final range	−0.3 to +0.7
Scale, final	absorbance

After zeroing the instrument, change the range to −0.3 to +0.7 and the scale to absorbance with the cuvettes in the cuvette holder. The pen will now automatically move to 0.3 absorbance on the chart paper. If the pen is not exactly at 0.3 absorbance, adjust it with the 100 per cent control knob. Record the spectra from 400 mμ to 230 mμ using the previously specified conditions.

Analysis of Unknown Solutions (Macromethod).—Pipette 1 ml of serum (urine or use a weighed portion of tissue homogenate) into a separatory funnel. Add 1 ml of 6 N hydrochloric acid and 50 ml of chloroform. Extract the acidified material for 5 minutes. Filter the chloroform layer into a clean dry separatory funnel, discarding the aqueous phase. Add 7 ml of 0.45 N sodium hydroxide to 45 ml or more of the chloroform solution of salicylate and back extract the chloroform phase for 5 minutes. Discard the chloroform layer and centrifuge the aqueous phase. Place two 3 ml portions of the aqueous phase into two silica cells. To one cell add 0.5 ml of 0.45 N sodium hydroxide and to the other add 0.5 ml of 16 per cent ammonium chloride. Mix well, cover, and read differentially using the ammonium chloride treated sample in the reference side.

Analysis of Unknown Solutions (Micromethod).—Pipette 0.1 ml of serum (urine or a weighed portion of tissue homogenate) into 10 ml of chloroform. Add 0.1 ml of 6 N hydrochloric acid and extract for 5 minutes. Centrifuge, carefully aspirate off the upper layer, and discard the aspirated material. Pipette 1.0 ml of 0.45 N sodium hydroxide into a 9 ml aliquot of the chloroform solution and back extract for 5 minutes. Centrifuge and carefully remove the lower layer using a long needle on a syringe. Pipette 0.4 ml aliquots of the alkaline extract into two microcuvettes and then add 0.05 ml of 20 per cent ammonium chloride to one cuvette and 0.05 ml of 0.45 N sodium hydroxide to the other cuvette. Mix and read differentially using the conditions previously described, and with the smallest masking slit of the DK 2 filter holder turned parallel to the instrument slit.

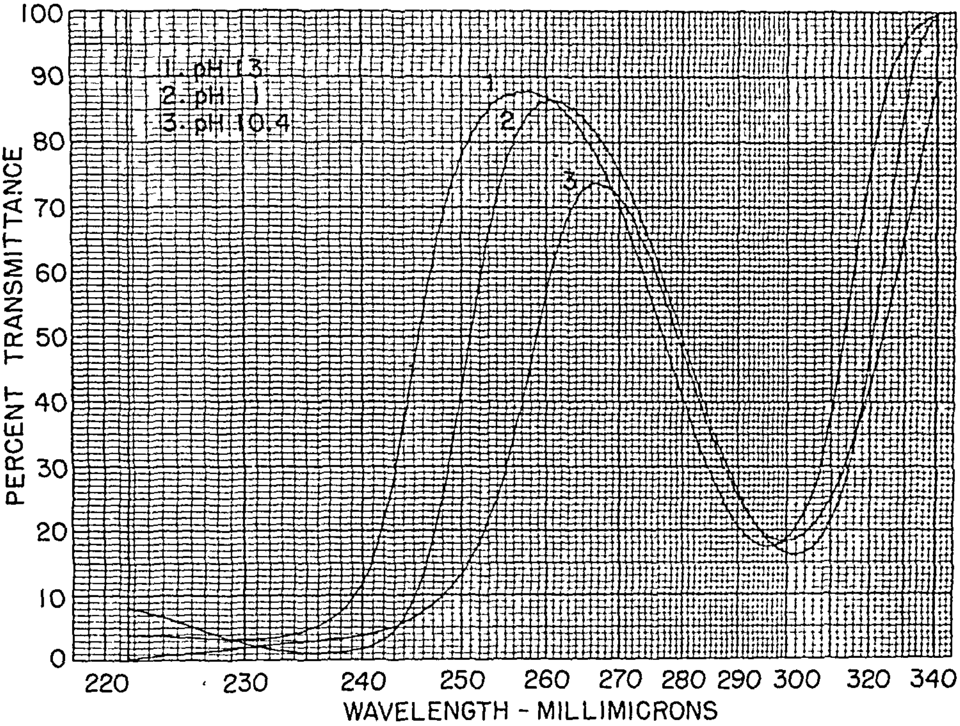

Fig. 1.—Wave length per cent transmittance spectra for salicylate at various pH levels.

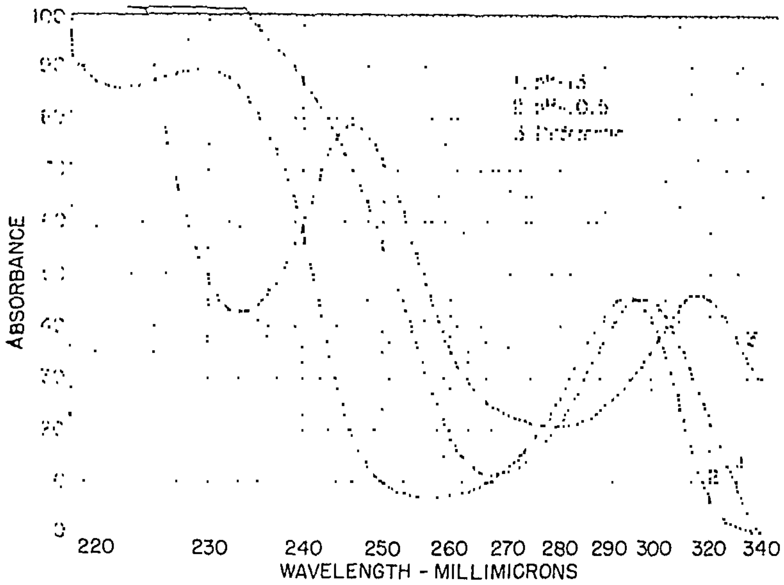

Fig. 2.—Normal and differential spectra of salicylate at pH 10.5 and pH 13.

RESULTS AND DISCUSSION

In order to determine the two pH levels which would give adequate analytic sensitivity as well as distinguishing qualitative characteristics a number of spectra were graphed at various pH levels. The extremes are shown in Fig. 1. These transmittance wave length curves were made with 4.3 μg of salicylate per millimeter and recorded against their appropriate blank. It is quite evident that the best differential curve could be obtained by the subtraction of the readings of Curve 3 from Curve 1. Curve 1 was carried out in 0.45 N NaOH, Curve 2 in 0.1 N H_2SO_4, and Curve 3 in NaOH and NH_4Cl buffer of pH 10.4. These happen to be ideally suited since they are also the best pH's for the similar determination of barbiturates.

Fig. 2 shows the spectra obtained with salicylates using the two pH levels decided upon. Curve 1 shows the pH 13 graph, Curve 2 shows the pH 10.5 graph, and Curve 3 shows the composite spectrum obtained by using the differential characteristics of the instrument to subtract one curve from the other as described in materials and methods. The two maxima occur at 318 and 246 mμ while the two minima occur at 283 and 233 mμ, respectively. Since both cuvettes contain equal aliquots of the same extract material and are distinguished from each other only by pH difference, interference from extraneous ultraviolet absorbing material can only occur if the interfering compound also shows a similar hypsochromic spectrum shift at the two pH values employed. This represents a unique advantage over normal ultraviolet analysis of biologic material since there are many compounds capable of exhibiting ultraviolet spectral characteristics which could show an additive error on the desired constituent and which could not show the characteristic shift with pH. Thus, graphing one against the other would yield a straight line for this material with no interference on the differential spectrum.

Fig. 3 shows a series of differential absorbance wave length spectra for various concentrations of salicylate. The range extends from 4 mg per 100 ml for Curve A to 55 mg per 100 ml for Curve F. It can be seen that the 318 mμ peak could be usefully employed for high concentrations of salicylate whereas the peak at 246 mμ would be suitable for lower concentrations of salicylate. The latter peak has a sensitivity of 3 times that of the peak at 318 mμ. The minimum at 283 mμ is of a lower order of sensitivity than is the maximum at 318 mμ and could be used for salicylates above the 70 mg per 100 ml level.

Fig. 4 shows the calibration curves obtained using the absorbance differences at the 3 wave lengths. The 246 mμ calibration plot is sensitive and useful from 0 to 20 mg per 100 ml while the 318 mμ plot covers the range of 0 to 70 mg per 100 ml. Above 70 mg per 100 ml, one could use the 283 mμ curve although the order of sensitivity is lower.

Table I shows a series of representative recoveries may by extracting absolute amounts of salicylate from pure solution as well as from additions to sera which had been shown to be salicylate free. In comparing the differential

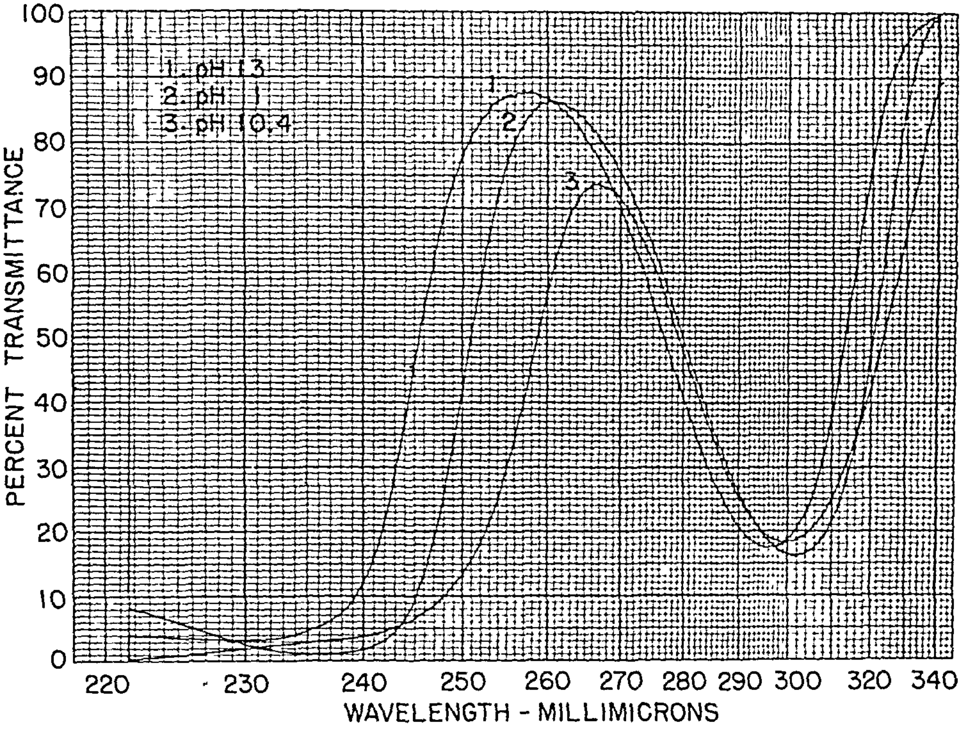

Fig. 1.—Wave length per cent transmittance spectra for salicylate at various pH levels.

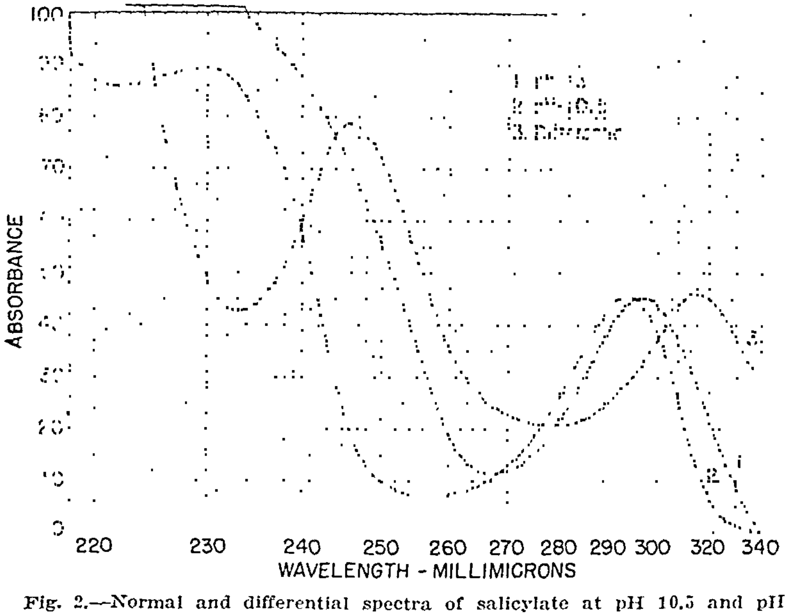

Fig. 2.—Normal and differential spectra of salicylate at pH 10.5 and pH 13.

spectra of salicylate extracted from sera and water, a reasonable similarity was obtained between the pure solution and the matrix investigated. The accuracy of the recovery of these comparisons and those in Table I were excellent.

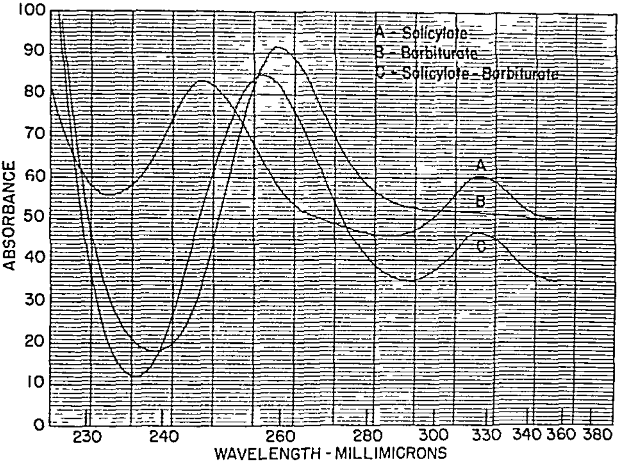

Fig. 5.—Curve A, salicylate differential spectrum. Curve B, seconal differential spectrum. Curve C, differential spectrum of a mixture of barbiturate and salicylate.

TABLE I. RECOVERY OF ABSOLUTE ADDED AMOUNTS OF SALICYLATE FROM WATER AND SERUM

SAMPLE	SALICYLATE PRESENT (MG./100 ML.)	SALICYLATE FOUND (MG./100 ML.)
Serum A	10.0	10.0
Serum B	20.0	21.0
Serum C	30.0	30.2
Serum D	40.0	40.6
Serum E	15.0	14.0
Serum F	10.0	10.5
Serum G	14.0	14.5
Serum H	20.0	20.8
Serum I	30.0	28.0
Serum J	40.0	44.6
Standard 1	10.0	10.0
Standard 2	10.0	10.2
Standard 3	20.0	19.7
Standard 4	30.0	29.4
Standard 5	30.0	29.2
Standard 6	40.0	43.2

Curve B of Fig. 5 shows the characteristic nature of the composite spectrum one obtains in this case by differential spectrophotometric means with a common barbiturate derivative. Curve A represents a similar consideration for salicylic acid, i.e., a composite differential spectrum with a pH 10.5 solution of salicylate in one cuvette and a very alkaline solution of salicylate in the other (0.45 N NaOH). It would not be difficult to recognize whether one or the other were

NATURALLY ACQUIRED TOXOPLASMOSIS IN THE GRAY SQUIRREL, SCIURUS GRISEUS, AND ITS BEARING ON THE LABORATORY DIAGNOSIS OF RABIES

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IN NOVEMBER, 1957, the carcass of a gray squirrel (*Sciurus griseus*) was sent to this laboratory with a request that the brain be examined for evidence of infection with rabies virus. The animal had been noted to behave in an abnormal manner and had bitten a child. Because of this episode, the animal was impounded; shortly after its capture, it became comatose and died on the following day.

Laboratory examination revealed no evidence that the squirrel had succumbed to rabies but, rather, that death might possibly have been due to toxoplasmosis. Findings bearing upon this latter point form the basis of this report, which is presented because of the almost complete absence of information on naturally occurring Toxoplasma infection in squirrel species, and because toxoplasmosis is seldom considered in the examination of tissue of wild animals showing overt evidence of C.N.S. disease.

MATERIAL AND METHODS

Preparation of Inoculum.—A small portion of the squirrel brain was weighed and ground manually in a mortar until a fine paste was obtained; sufficient 0.85 per cent saline solution was added incrementally to give a 10 per cent suspension. This suspension was centrifuged horizontally at 2,500 r.p.m. for 10 minutes and the supernatant fluid carefully removed. To each 0.9 ml. of fluid was added 0.1 ml. of an antibiotic solution containing 10,000 units per milliliter of penicillin G and 50,000 units per milliliter of streptomycin sulfate. The mixture was incubated at 4° C. for approximately one hour and then inoculated intracerebrally in 0.03 ml. amounts into mice 3 to 4 weeks old.

Microscopic Examination.—Smear and imprint preparations were made from the squirrel brain, utilizing tissue from the region of Ammon's horn. The preparations were stained by Seller's method, which is routinely employed in this laboratory for the staining of Negri bodies. Subsequently, when the presence of toxoplasms was suspected, additional smear and imprint preparations were made and stained by the Giemsa method.

RESULTS

Comprehensive examination of numerous microscopic fields of the slides stained by Seller's method failed to reveal the presence of any structures identifiable as Negri bodies. Examination of these slides with an oil immersion objective revealed the presence of a number of large round structures about 30 to 50 μ in diameter and containing numerous small bodies of 2 to

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present if they were present as a single contaminant of serum or tissue. If one were to be confronted with the prospect of both being present at the same time, Curve C would be the resultant composite spectrum obtained using the described differential technique. The height of the first differential salicylate peak appears unimpaired by the contaminant whereas the next two absorbance peaks, both negative and positive, are so affected as to be inaccurate. Reextraction of a second portion of the investigated material at pH 7.4 leaves the salicylic acid unextracted while the barbiturate is completely removed to the chloroform phase. Thus, one is able to analyze a mixture of both salicylate and barbiturate if they are present in the same physiologic matrix. More commonly they occur as individual entities, and the procedure is simplified. However, salicylate can be analyzed in the presence of barbiturate with good accuracy, and this would be more difficult to accomplish by conventional ultraviolet spectrophotometry.

SUMMARY

A procedure has been described for the determination of salicylate which involves the use of the differential characteristics of an automatic ratio recording spectrophotometer. The technique obviates normal blanking and eliminates interference from ultraviolet absorbing material. The instrument automatically subtracts two spectra of salicylate, one from the other, after a hypsochromic shift with a pH change and draws a composite spectrum. The analysis range is wide, the procedure is applicable to 100 μl samples, and barbiturate, when present, can be determined regardless of whether salicylate is also present or entirely absent.

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preparations from the brain of both animals showed the presence of toxoplasmal pseudocysts. Fig. 2 shows the structures seen in the brain of the first mouse.

DISCUSSION

Since the original reports of Nicolle and Manceaux[1] from North Africa and of Splendore[2] in Brazil describing Toxoplasma as a new protozoon from the gondi (*Ctenodactylus gondi*, a rodent) and from the domestic rabbit respectively, the parasite has been described from a wide variety of mammalian hosts, from some marsupials and from certain avian species. Many of these descriptions of natural infection are based on morphologic observations and hence may be open to question; although the appearance of *T. gondii* is rather distinctive, an unknown organism cannot be identified definitively as *T. gondii* on the basis of morphology alone.[3] In addition to possessing the morphologic and tinctorial characteristics of *T. gondii*, the unknown microorganism should be shown to be an obligate intracellular parasite, to possess pathogenicity for mice and other laboratory species, and to be immunologically related to known strains of *T. gondii*. Unfortunately, because of the pressure of other and more urgent work, no attempt was made to fulfill these criteria. One important consideration, based on the results of inoculation in the first passage mice, and perhaps unwarranted, was that *T. gondii* may acquire pathogenicity only after prolonged passage at frequent intervals and that some strains, even after a year or more of continual passage at frequent intervals, still retain minimal levels of virulence.[4-6] Thus, Pope and associates[5] describe a strain which produced only inapparent infections in mice during the first 64 intraperitoneal passages; thereafter the virulence for mice gradually increased, and from the eighty-fifth passage onward the mortality was 100 per cent.

Although the diagnosis of toxoplasmosis in the tree squirrel, *Sciurus griseus*, reported upon here is based entirely upon the tinctorial and morphologic characteristics of the microorganism seen in tissue preparations, we considered it desirable to record the findings since the only other instance of natural toxoplasmosis in the squirrel (red squirrel) of which we are aware is that recorded by Coles in 1914. In addition, the experience recorded here suggests that *T. gondii* should be considered as a possible etiologic agent in the production of those symptoms that lead to subsequent examination of the animal for rabies virus infection and might especially be considered when the results of such examination prove negative. This possibility finds support in recent reports[8, 9] from North Africa that the squirrel *Citellus citellus* is highly susceptible to experimental toxoplasmosis.

SUMMARY

The brain of a California gray squirrel (*Sciurus griseus*), a tree-living species, was examined for rabies virus because the animal had been observed to behave in an abnormal manner and had bitten a child. Microscopic examination of smear and imprint preparations of the brain was negative for

Negri bodies but structures with the typical morphologic and staining characteristics of *T. gondii* were encountered. This is believed to afford at least presumptive evidence of natural infection with *T. gondii* in a squirrel.

The excellent technical assistance of Mr. Koichi Nakamura is gratefully acknowledged.

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CLINICAL AND EXPERIMENTAL

THE FINDING OF URINARY PROTEOSE IN CERTAIN RENAL DISEASES

ISOLATION AND IDENTIFICATION

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THE most common protein precipitants employed in the routine evaluation of proteinuria are sulfosalicylic acid, trichloroacetic acid, and heat and acetic acid. While it has been known for many years that there are qualitative differences in the proteins of urines from patients with varied diseases,[1] it has not always been possible to differentiate by simple chemical means between the proteinuria of various types of active renal disease and that of congestive heart failure, pregnancy, and other extrarenal proteinurias by these precipitants or indeed by more elaborate techniques of physical chemistry.

In the routine analysis of urines from nephritic and cardiac patients one of us (J. M. L.) noted that the initial urines of a patient with active glomerulonephritis confirmed by biopsy yielded heavy precipitates on first boiling, followed by acidification but much less to no precipitate when the order was reversed, that is, the urine *first acidified with acetic acid and then boiled*. (The details of clinical and laboratory findings on this patient are given in the Appendix.) The protein-containing urines from certain cardiac patients and from certain pregnant women examined at the same time did not reveal this phenomenon.

The phenomenon suggested presence of a heat-resistant, water-soluble substance, protein-like in nature.

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(8) contains approximately 30 mg. of bound hexose and 50 mg. of bound hexosamine per 100 mg. of nitrogen; and (9) reported by Dr. R. J. Winzler as giving a strongly positive precipitin reaction with orosomucoid antisera. (This may be an instance of a cross reaction reported for proteins of high carbohydrate content.)[7]

The solubility properties displayed by the substance isolated from the urine fulfills the criteria set forth in the definition for proteoses given in the introduction.

Further physical and chemical studies are being conducted on this proteose and will be reported in a future communication.

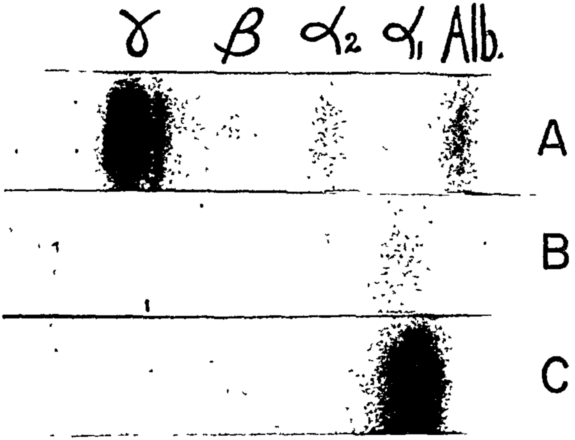

Fig 1—Patient J McQ Strip A serum electrophoresis with veronal buffer at pH 8 6 0 1 ionic strength and stained with bromphenol blue Strips B and C, isolated proteose electrophoresis under the same conditions, but with B stained with bromphenol blue and the C with periodic acid leucofuchsin (Schiff reaction). Note correspondence of position of chief component in B and C with the chief (faster) component in C of Fig 3.

Patients.—Patients with proteinuria were selected from the wards at Cook County Hospital, and the urines from nephritic patients who had been followed for periods up to 2 years in the Research Nephritic Clinic at Hektoen Institute were studied. The usual methods of clinical classification were supplemented by renal biopsy in 13 of the 50 patients studied.

Acute Glomerulonephritis (Acute Phase).—A detailed report of the clinical, laboratory, and renal biopsy findings is given in the appendix for Patient R B. on whom serial studies have been made. Analysis of his urine some 10 days after sore throat and about a week after onset of edema revealed a 4+ reaction with 5 per cent sulfosalicylic acid, and a reaction of the same intensity with heat, but no precipitate formed when the urine was first acidified with acetic acid and heated to boiling. The proteose reaction was 3+.

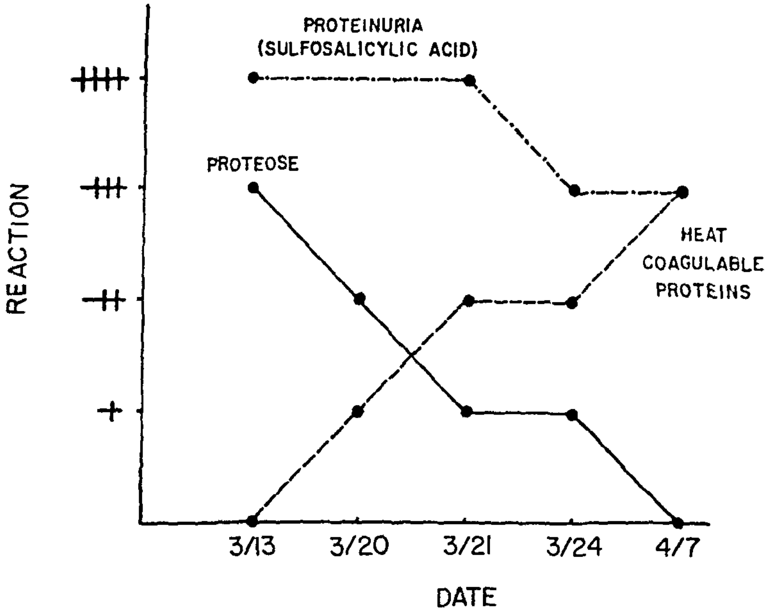

Fig. 2.—Patient R. B. For identification of dates relative to clinical course, sore throat appeared about 3/4 and generalized edema appeared about 3/8, was almost gone about 3/24, and completely gone 4/7 (see text and Appendix).

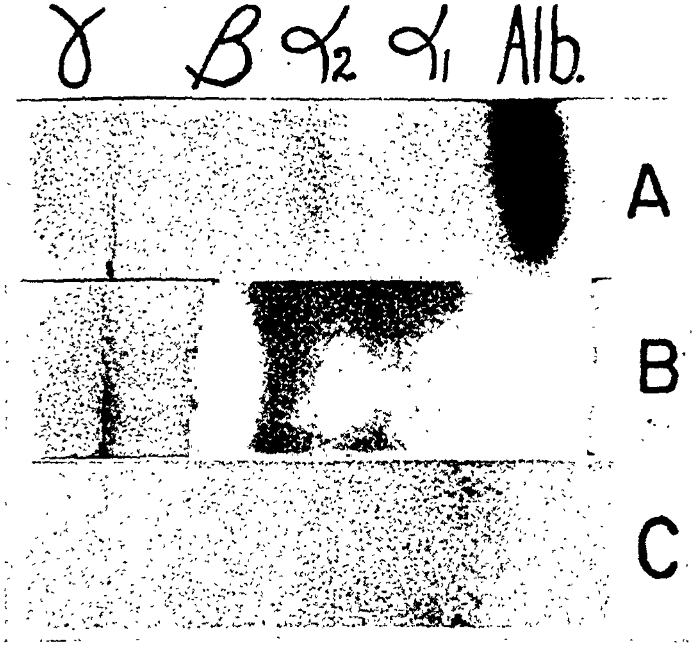

Fig. 3.—Patient R. B. Strips A and B, serum and urine electrophoresis, respectively, with veronal buffer at pH 8.6, 0.1 ionic strength, and stained with bromphenol blue. Strip C, urine electrophoresis under the same conditions but stained with periodic acid leucofuchsin (Schiff reaction). Compare C with B and C of Fig. 1.

One week later, when edema was almost gone, the urine still gave a 4+ reaction with sulfosalicylic acid, but a precipitate now appeared when the acidified urine was heated to boiling The proteose reaction was less about 2+ Subsequent analyses revealed that the detectable heat coagulable proteins increased while the proteose content decreased until about a month after onset (with patient asymptomatic and edema free) there were only heat coagulable proteins with no proteose to be detected These findings are illustrated in Fig 2

Serum and urine protein electrophoreses were performed by methods previously described from this laboratory,[8] and the patterns of the urine disclosed a trailing of the albumin corresponding to the alpha 1 position An electrophoretic strip of urine collected some 2 weeks after onset of edema, when treated with the periodic acid Schiff reagent revealed a positive reaction at the alpha 1 position (Fig 3)

TABLE I HEALED ACUTE GLOMERULONEPHRITIS

PATIENT	ORIGINAL DIAGNOSIS	URINE STUDY			
		TIME AFTER ACUTE EPISODE (MONTHS)	SULFOSALICYLIC ACID	ACETIC ACID AND HEAT	PROTEOSE
M M *	Biopsy	8	0	0	0
J G †	Biopsy	12	0	0	0
P Sn ‡	Biopsy	11	0	0	0
R G §	Clinical	17	0	0	0
M P §	Biopsy	5	0	±	0
A W ‖	Biopsy	17	+++	+++	0

*Repeat biopsy at time of the above urine study showed glomerular healing but also a persistent interstitial nephritis which in certain areas was severe enough to be associated with tubular atrophy and as many as 4 000 colonies of mixed flora of gram positive and gram negative organisms per milliliter of catheterized urine (It is of interest that despite initial recovery of Type 12 beta hemolytic streptococci the antistreptolysin and antistreptokinase titers never rose above normal limits)

†*This patient s antistreptolysin maximum was 833 with rapid subsidence after acute* episode While some 6 months after this episode there was transitory suspicious evidence of a superimposed pyelonephritis (by physical examination and examination of urine sediment and bacteriology) at the time of the study of urine here reported this had subsided under therapy

‡Renal biopsy 2 months after acute episode showed in addition to hypercellularity of glomeruli and an occasional inflammatory cell some early lobulation and adhesions Repeat biopsy 4 months after acute episode showed regression of the former glomerular changes but persistence of the latter at a time when the patient by ordinary clinical criteria was well and urine showed only evanescent proteinuria and an occasional cast At the time of urine study here reported these had disappeared

§Multiple urine examinations subsequent to this study disclosed complete absence of protein and pathologic formed elements

‖Type 12 beta hemolytic streptococci were recovered at the time of acute episode This
aximum was 330 at that time and has fluctuated around the 200
nuria has persisted it is an intermittent finding in monthly ex-
been absent from sediment for at least 6 months prior to the urine
the only remaining pathologic formed elements are limited to

Acute Glomerulonephritis (Apparent Clinical Recovery)—In Table I are given urine protein and proteose studies for 6 patients whose hemorrhagic glomerulonephritic episodes occurred over 5 months previously Diagnosis was confirmed by renal biopsy in 5 (For details of 2 follow-up renal biopsies, see footnotes to Table I) In the urines of all 6 patients no proteose was found, in 4 proteinuria had also disappeared and in 2 patients transient proteinuria persisted despite absence of hematuria, clinical well being, and creatinine clearances which, in serial follow up studies, stabilized close to normal limits

in contrast to a group of 10 patients who, though comparable in degree of qualitative proteinuria, showed no evidence of proteose in the urine. Of these, 8 were in congestive heart failure (2 were classified as cor pulmonale, 2 as arteriosclerotic heart disease, and 4 as hypertensive heart disease). The 2 other patients in this group carried the diagnoses of metastatic carcinoma and chronic relapsing pancreatitis, respectively.

Miscellaneous Proteinurias.—One patient with a clinical diagnosis of lupus erythematosus (whose renal biopsy showed glomerular changes consistent therewith) showed *proteosuria in association with the proteinuria. Two patients* with the same diagnosis and in the absence of evident renal involvement showed no proteosuria or proteinuria. In addition, 3 patients with multiple myeloma were studied, 2 showed evidence of Bence Jones proteinuria, and one showed proteinuria in the absence of Bence Jones proteinuria. All 3 patients showed positive qualitative tests for proteosuria. The proteose findings may be explained by the presence of Bence Jones protein which is proteose in nature.[9]

DISCUSSION

The literature is replete with investigations of proteinuria, and innumerable studies have been conducted on sera and urines of nephritic patients.[10, 1] The problem of the nature of the nephritic proteinuria has been approached essentially from these directions: (1) the qualitative whereby the stability of a gold sol was studied with the urines from patients with various pathologic conditions,[1] and the specific serologic reactions whereby sera drawn during the edematous stage of nephrosis were reacted with antibodies from rabbits hyperimmunized with serum globulin from nephritic patients[1]; (2) the quantitative whereby proteins were fractioned into albumins and globulins, studying their ratios and comparing the findings with that of sera.[11, 1] Most recently, electrophoresis by the paper strip method has been employed for the separation and quantitation of serum and urinary proteins in nephritis.[1]

By the usual methods for urinary protein precipitation, such as sulfosalicylic acid or trichloroacetic acid, the proteose also precipitates. By these techniques, it was impossible to differentiate the proteose from the albumin and globulin. These techniques overlooked the possibility of the presence in urine from nephritic patients of a heat-soluble protein which is precipitated with sulfosalicylic acid and trichloroacetic acid but is released from the precipitate on boiling and then recovered in the filtrate: a protein which is left in solution after the coagulation of albumin and globulins by acidifying and boiling the urine. These were the properties which led us to the isolation of the proteose.

Beckman and associates[1] reported the isolation of a protein soluble in trichloroacetic acid. No mention of the etiology of the proteinuria is made. However, a comparison of the properties of their protein with the proteose isolated by us from the urine of nephritic patients does not reveal identity. While the Beckman protein was not precipitated by 0.25 M trichloroacetic acid, the material here reported is so precipitated. The Beckman protein was precipitated by heat and acetic acid while the proteose reported here is soluble, and this is the basis for the separation of the proteose in our proposed diagnostic test for active renal disease. While the proteose is not precipitated until full saturation with ammonium sulfate, the protein of Beckman and associates was precipitated by half-saturated ammonium sulfate. Electrophoretically, the mobility was similar. The authors do not report whether their protein contained carbohydrate. The proteose reported here gives a strongly positive test for carbohydrate with the periodic acid-Schiff reagent (Fig. 1).

Popenoe[18] reported an investigation of the trichloroacetic acid-soluble protein of urine reported by Beckman and co-workers[17] and concluded (1) that the principal acid-soluble protein of children with nephrotic syndrome is indistinguishable from the alpha-1 acid glycoprotein isolated by Schmid[19] from normal human plasma by electrophoresis, by analysis for hexose, hexosamine, and nitrogen, and by a specific precipitin test and (2) that it is not unique to pathologic urine. The glycoprotein reported by Popenoe[18] did not form a precipitate on heating in dilute acid or on half-saturation with ammonium sulfate and was soluble in 0.25 M trichloroacetic acid, 1.8 M perchloric acid, and in 0.6 M sulfosalicylic acid fulfilling the criteria for the purified acid glycoprotein of normal plasma.[19, 20] The proteose isolated by us from the urine of nephritic patients is precipitated by all the above-mentioned acids of the stated concentrations. The comparison of the properties of the materials reported by Beckman and associates,[17] Popenoe,[18] and of the proteose reported here is summarized in Table II.

TABLE II

REAGENTS	BECKMAN AND ASSOCIATES[17]	POPENOE[18]	MATERIAL HERE REPORTED
0.25 M trichloroacetic acid	Soluble	Soluble	Precipitated
Acetic acid and heat	Precipitated	Soluble	Soluble
Salting out with ammonium sulfate	Half saturation	Full saturation	Full saturation
1.8 M perchloric acid		Soluble	Precipitated
0.6 M sulfosalicylic		Soluble	Precipitated

In a most recent and extensive study of the protein-bound carbohydrates in human serum and urine in proteinuria, Laurent[21] reported that in pathologic sera a wide range of quotient values was found indicating the presence of abnormal proteins and/or pathologically increased amounts of normally occurring protein fractions with carbohydrate quotients deviating from the average values. In proteinuria, the albumins and globulins of the urine contained hexose in approximately the same amounts as the serum. There were no salient differences between the bound-carbohydrate quotients of the sera investigated and between the corresponding serum and urine fractions. The results from his study of 13 normal human sera, 86 sera obtained from patients with kidney disorders and miscellaneous conditions, and 44 urine samples from patients with proteinuria of varied origin led Laurent to the conclusion that no loss of bound-carbohydrates occurred during the passage of protein through the kidneys.

Goettsch and Lyttle[16] reported in their precipitin studies in nephrosis and nephritis that the altered protein in the globulin fraction of 3 patients, when injected into the rabbit, formed antibodies which did not react with normal globulin, the antibodies reacted with nephrotic sera drawn during the edematous stage of nephrosis but not with the sera of healed nephrotic patients These temporal relations are analogous, although not identical, with the sequences noted above for urinary proteose in the serial study of Patient R B (acute glomerulonephritis) We have no comparable sequential studies in our chronic glomerulonephritic group, in part, because it is chiefly adult, and "healing" in an adult glomerulonephritic nephrotic patient is rare *

Our preliminary studies lead us to believe that the occurrence of urinary proteose (in contradistinction to protein), may be correlated with active parenchymal renal inflammatory changes Proteosuria per se does not yet, in our experience, distinguish between glomeruli and tubules as primary foci of inflammation or between direct bacterial and autoimmune pathogenesis The absence of proteosuria in our proteinurias whose pathogenesis is probably not correlated with parenchymal inflammation leads us in rather old fashioned terminology, to the suspicion that while proteinuria may be transudative or exudative, proteosuria is clinically indicative of an exudative process

SUMMARY

1 The isolation of a proteose from the urine of nephritic patients is reported This proteose is electrophoretically characterized by its position predominantly at alpha 1, extending almost to the alpha 2 position, and yields a strongly positive carbohydrate reaction with the periodic acid Schiff reagent

2 A simple test based on the solubility of this proteose is presented, with evidence (based on a study of 50 patients in diagnostic categories including acute active and "healed" glomerulonephritis, chronic glomerulonephritis pyelonephritis, diabetic nephropathy, and congestive heart failure) suggesting its utility in the clinical differentation of active parenchymal renal inflammation from proteinurias produced by other mechanisms

APPENDIX

R B (C C H 58 17226), an 18 year old Puerto Rican male, was admitted with chief complaint of periorbital edema of 4 days' duration One week before admission he had a sore throat which had subsided by the time of edema appearance He denied visible hematuria, headache, prior episodes of edema, or any other phenomena suggestive of glomerulonephritis or pyelonephritis

On physical examination the only significant abnormal finding was a minimal generalized puffiness noted particularly in the face, hands sacrum and ankles Specifically, blood pressure was within normal limits as were the fundi and heart size

The initial opinion of the renal team favored acute glomerulonephritis as a clinical diagnosis, however, in the absence of gross hematuria, one member believed the proper classification to be an exacerbation of a chronic membranous glomerulonephritis

Although his initial urine was not grossly bloody, on microscopic examination it was loaded with red cells and, in addition, contained a few white cells and granular and hyaline casts in the centrifuged sediment Neither white nor red cell casts were noted in the first 2 weeks of his course, and there were no white cell clumps

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*Ongoing serial studies in collaboration with Dr Aaron Crossman of Cook County Children s Hospital are aimed at elucidation of this point

His initial serum albumins were 3.6 Gm. per cent, total globulins 1.5 Gm. per cent, gamma globulins 1.1 Gm. per cent, and cholesterol 147 mg. per cent.

An initial nonprotein nitrogen of 70 mg. per cent had returned to 32 mg. per cent at the end of the first week of hospitalization and his edema had disappeared. At this time, his 24-hour proteinuria amounted to 9.1 Gm.

At the end of his second hospitalized week, proteinuria amounted to 6.1 Gm. per 24 hours, creatinine clearance was 86 ml. per minute, serum creatinine 1.0 mg. per cent, total serum lipids were 580 mg. per cent, lipid phosphorus 9 mg. per cent, serum mucoprotein was 81 mg. per cent, and gamma globulins were 0.85 Gm. per cent.

His initial urine culture showed no growth. His initial throat culture showed no beta hemolytic streptococci. However, while his initial antistreptolysin titer taken at the end of 2 weeks was 250, by the fourth week it had arisen to 333. The corresponding antistreptokinase values were 160 and 320, respectively. By the sixth week, the antistreptolysin titer had fallen to 250, and it remained at this level or below for the remainder of his course. His antistreptokinase titer, which was 160 2 weeks after admission, rose to 320 4 weeks after admission and thereafter returned to its initial normal level.

Renal biopsy, carried out one month after admission, was consistent with a diagnosis of proliferative glomerulonephritis, with findings suggestive of a chronic membranous process at a minimum. (Detailed report of histology is in preparation for publication in collaboration with Dr. P. B. Szanto.) Culture of this biopsy was sterile.

At this time, multiple urine examinations continued to show 4+ proteinuria; 24-hour proteinuria amounted to 3.4 Gm. Microscopic hematuria had all but disappeared and the formed elements of the sediment were limited to a few white cells and hyaline casts.

When seen up to 3 months after admission in the Research Renal Clinic, he continued to be asymptomatic and normotensive with normal serum cholesterol, lipid phosphorus, total lipids, and a creatinine clearance remaining in the 90's. However, a 1+ to 2+ proteinuria and minimal microscopic hematuria has persisted. On one occasion, white cell clumps were seen in the urine. Two urine cultures were sterile; a third showed nonhemolytic staphylococci (albus) with a colony count of only 440 per milliliter of urine.

We are indebted to Warden Fred Hertwig, Cook County Hospital, and to Drs. Leonard H. and Louis D. Weissman, Medical Research Foundation, for the provision of basic facilities for protein research at the Hektoen Institute.

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contained a pyroglobulin[5] The macroglobulin is included in the pyroglobulin fraction, since electrophoresis of the serum, after removal of pyroglobulin, no longer showed the presence of macroglobulin

Serum D contained a macroglobulin diagnosed on the basis of water dilution, temperature viscosity index, and ultracentrifugal analysis The latter again indicated 3 components, with sedimentation constants of 4 5S, 17S, and 23 6S *

Electrophoresis—The electrophoresis was conducted by the fluid film method, which has been described previously[6] A veronal buffer of pH 8 6 was used at the following concentrations 0 01 M, 0 02 M, 0 033 M, 0 066 M, 0 1M, and 0 15 M At each concentration, all 5 sera were migrating simultaneously in the same buffer solution, thus tending to cancel out possible variations that could affect the comparison of different sera at the same ionic strength The electrophoresis was conducted for a period of 4½ hours at 160 volts, corresponding to a potential gradient of 5 0 volts per centimeter

RESULTS

The electropherograms of the 5 sera, at each of the 6 buffer concentrations, are shown in Fig 1 Densitometer curves (optical density of the stained electropherogram along the migration path) for the normal controls were obtained with a Kelab densitometer and are presented in Fig 2 The relative mobility of the 4 abnormal globulins was determined from the position at which the outside edge of the migrating band was parallel to the direction of the electric field Each abnormal globulin is designated in Fig 2 by a bar indicating its position when the normal and abnormal electropherograms are superimposed

It is seen that mobility varies with buffer ion concentrations For example, as the buffer concentration decreases, globulin A moves progressively closer to the rear or toward the cathode in the same electropherograms and also as compared to the γ globulins of the normal control, Fig 2 The mobility of globulin B, however, compared to the mobility of normal β_1 globulin appears constant The macroglobulins remain at the point of origin in the 0 01 M and 0 02 M buffers In the 0 033 M buffer, macroglobulin C begins to migrate from the origin, and a new faint band appears toward the cathode, suggesting an equilibrium between soluble and relatively insoluble fractions of this protein Macroglobulin D does not migrate until the buffer concentration reaches a value of 0 066 M The 6 normal electropherograms, corresponding to the curves of Fig 2, are reproduced schematically in Fig 3 The scales of the abscissa are altered as above

Since each macroglobulin does not migrate from the origin until the ionic strength reaches a specific value, the absorbance of sera C and D, each diluted 1 to 50 with veronal buffer, was measured at various buffer ion concentrations in a Coleman Universal spectrophotometer, 580 mμ As Fig 4 shows, an increase in turbidity, which can be correlated with an increasing particle size due to aggregation or decreasing solubility, occurs as the buffer concentration decreases The concentration at which the absorbance begins to increase is slightly below 0 04 M for serum C, and below 0 07 M for serum D These values correspond to the respective buffer concentrations at which the 2 macroglobulins begin to migrate from the origin

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*See footnote on opposite page

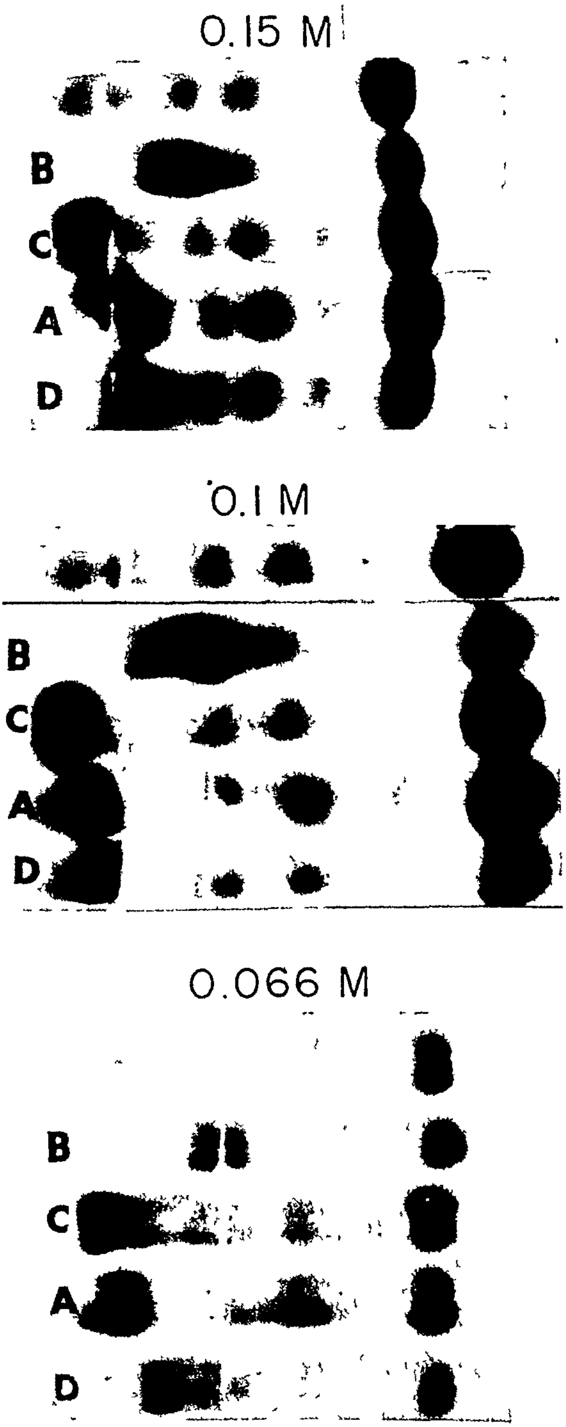

Fig 1*A*.

Figs 1*A* and 1*B*—Electropherograms at 6 indicated veronal buffer concentrations In each case, the top serum is the normal control, while the other sera are referred to in the text by the designated letter.

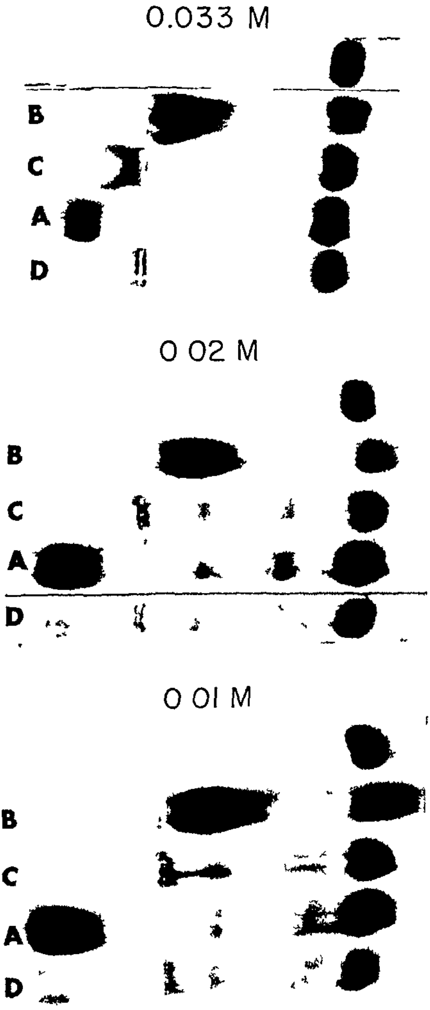

Fig 1*B*.—(*For legend see opposite page.*)

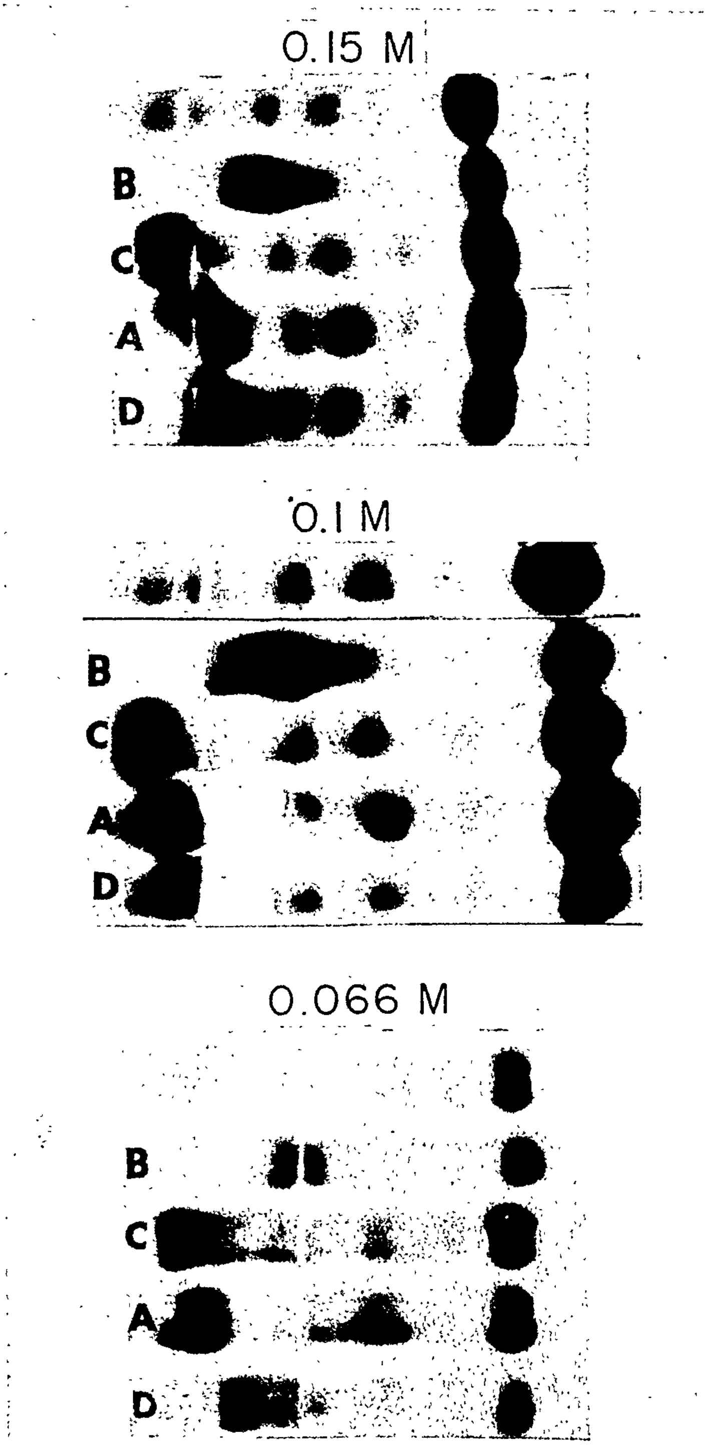

Fig. 1*A*.

Figs. 1*A* and 1*B*.—Electropherograms at 6 indicated veronal buffer concentrations. In each case, the top serum is the normal control, while the other sera are referred to in the text by the designated letter.

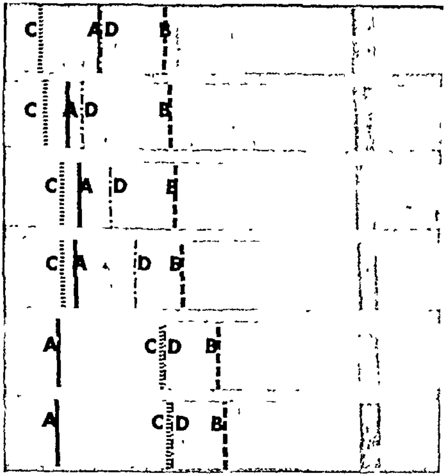

Fig 3.—Electropherograms corresponding to the curves of Fig 2, reproduced schematically to indicate the relative positions of the abnormal globulins

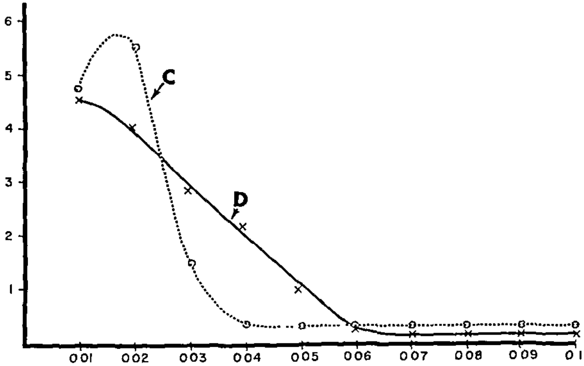

Fig. 4.—The absorbance of the 2 "macroglobulin-containing sera" after a fiftyfold dilution with buffers of varying concentration. Ordinate is absorbance and abscissa is buffer concentration The drop in the absorbance of serum C in most dilute buffer was due to the formation of a heavy precipitate.

DISCUSSION

The dependence of relative mobility of normal serum proteins upon ionic strength agrees with results obtained previously.[1] With a decreasing ionic strength, the results can be summarized as follows: (1) the relative mobility of α_1-globulin increases until, at a veronal concentration of 0.01 M, it becomes incorporated into the albumin band; (2) the relative mobility of the α_2-globulin also increases; (3) the width of the α_2-globulin band increases suggesting a dissociation into further components; (4) the β-globulin dissociates into a β_1- and a β_2-component. γ_1- and γ_2-globulin can be seen as distinct fractions only in the two highest buffer concentrations.

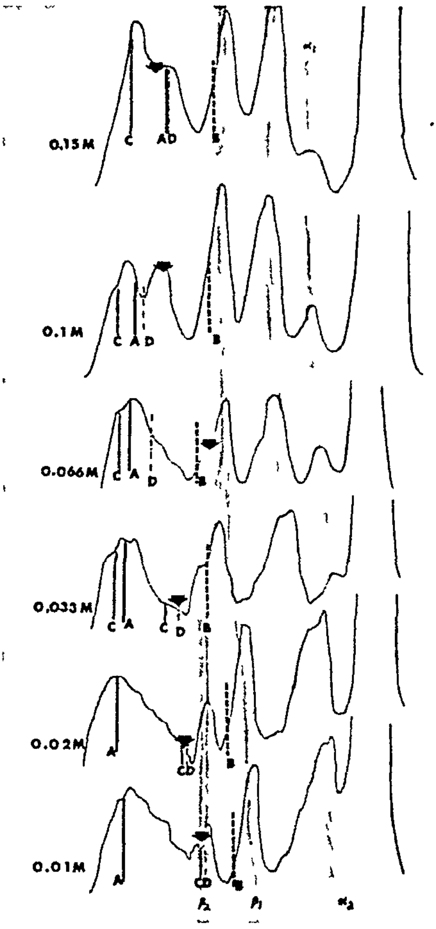

Fig. 2.—Densitometer curves of the normal control of Fig. 1. Relative mobilities of the abnormal globulin of each serum are indicated.

The alteration in the relative mobility and increasing separation of the various protein components with variation in ionic strength may be related to both the retarding effect of the ionic atmosphere and to binding effects between

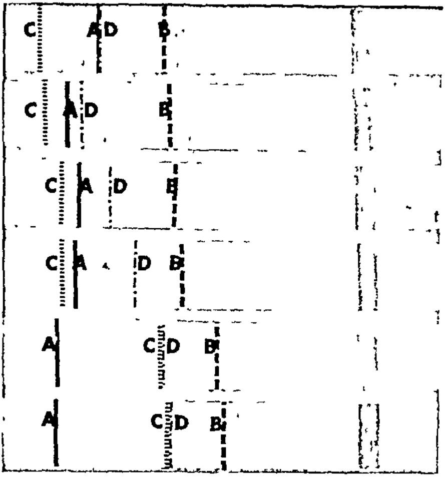

Fig 3 —Electropherograms corresponding to the curves of Fig 2, reproduced schematically to indicate the relative positions of the abnormal globulins

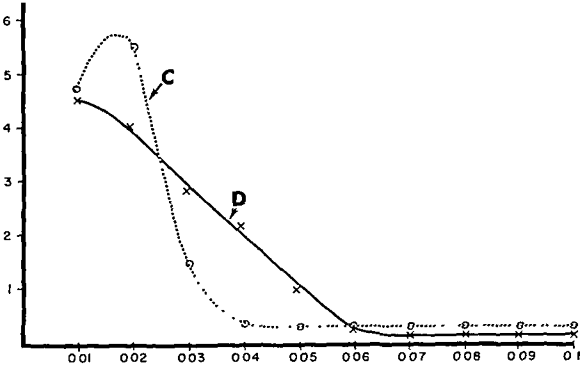

Fig. 4 —The absorbance of the 2 "macroglobulin-containing sera" after a fiftyfold dilution with buffers of varying concentration Ordinate is absorbance and abscissa is buffer concentration The drop in the absorbance of serum C in most dilute buffer was due to the formation of a heavy precipitate.

the proteins and buffer ions. The mobility (μ) of a protein molecule has been shown to be related to its charge (Q) by the expression[7]:

$$\mu = \left(\frac{Q}{6\,\pi\,\eta\,r}\right)\left(f\ (\kappa, r)\right)\left(\frac{1 + \kappa r_i}{1 + \kappa r + \kappa r_i}\right)\left(C\right)\left(f\ (\kappa, {}^{a}/_{b})\right)$$

where η is the viscosity; r is the effective protein spherical radius; r_i the average radius of the buffer ion; f (κ, r) a function correcting for field distortion by a large nonconducting particle; κ is the Debye function; C is a dimensional constant; and f (κ, a/b) is a function correcting for the asymmetry of a protein of axial ratio a/b.

It has been shown that the retarding effect of the ionic atmosphere due to the asymmetry function (κ, a/b) is quite marked and that its effect is not constant for a given shape, but increases with the ionic strength.[7] Since the serum proteins vary in regard to symmetry and axial ratio, their relative mobilities will thus be affected. A further cause for alteration of relative mobility with changing ionic strength may be unequal binding of buffer ions by the different proteins, which would thus influence the net charge, Q. Binding of buffer ions has been shown to be extensive, and to vary quantitatively, with individual proteins.[8-11]

Similar considerations would also apply to abnormal globulins. The relative shift in position of myeloma globulin A (Fig. 3) toward the rear of the gamma as the ionic strength is decreased illustrates that a myeloma globulin can exhibit the mobility of an "M" or a "gamma" type at one ionic strength, and the mobility of a "slow" type protein at another ionic strength.

The electropherograms obtained with the 0.15 M, 0.1 M, and 0.066 M buffers reveal an opposite effect of ionic strength on globulins A and D, A moving backward and C tending to move forward as the ionic strength decreases. This behavior is not exhibited by any normal protein visible in the same areas, which suggests that the abnormal globulins A and C differ, electrophoretically, from normal components. The possibility is not excluded, however, that corresponding normal components may exist, but in too low a concentration to be detected.

The macroglobulins, C and D, are the most grossly affected by ionic strength. This is due to aggregation or insolubility at lower buffer concentrations. The buffer concentrations at which the diluted serum begins to become more turbid (Fig. 4) corresponds to the buffer concentration at which each macroglobulin will no longer migrate from the point of origin (Fig. 1). The macroglobulin C shows an equilibrium between soluble migrating and insoluble nonmigrating fractions in the 0.033 M buffer. Although both macroglobulins are identical on the basis of ultracentrifugal analysis, the electrophoretic and solubility characteristics of each one are different, agreeing with the concept of "patient-specificity."[3] The heat sensitivity of globulin C is a further point of individual difference. The fact that the veronal buffer concentration at which macroglobulin D is sufficiently soluble to freely migrate lies close to 0.07 M, is especially important since paper electrophoresis is commonly conducted at a concentration of 0.05 M. Under such circumstances, the mobility of a macroglobulin may be altered by incomplete solubility. The concentration of buffer suitable for the electrophoresis of a given macroglobulin, without modification of mobility due to incomplete solubility, can readily be determined by the method described above

SUMMARY

Sera containing abnormal globulins have been subjected to electrophoresis in buffers varying in ionic strength It has been found that the mobility of an abnormal globulin, compared to the normal globulins of the same serum, may change with concentrations of buffer ions Possible reasons for this dependence are discussed

The two macroglobulins investigated did not migrate far from the origin in buffer concentrations below a critical value, specific for each protein This effect was found to be correlated with aggregation or insolubility of protein molecules A simple method is described to determine what buffer concentration may be suitable for the electrophoresis of any particular macroglobulin

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ALBUMIN METABOLISM IN NEPHROTIC ADULTS

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URINARY loss of albumin is a cardinal manifestation of the nephrotic syndrome.[1] The degree of hypoalbuminemia, however, has occasionlly seemed out of proportion to the albuminuria.[2] Recent studies have clarified the mechanism of the severe deficiency of serum albumin which may occur in nephrotic children. Using I^{131}-labeled serum proteins as tracers and immunochemical methods of assay, Gitlin and associates[3] have reported that in children with the nephrotic syndrome an abnormally increased fractional rate of catabolism of albumin, gamma globulin, and iron-binding globulin may accompany the urinary loss of these proteins and, thus, significantly contribute to their serum deficiencies.

The present report is a similar study of nephrotic adults to determine whether an abnormality of albumin metabolism occurred in the adult as well as in the childhood type of nephrosis.

METHODS

Three adult patients with latent nephrosis and 3 with overt nephrosis were studied. A summary of these patients is given in Table I.

Each patient was given a single intravenous injection of I^{131}-labeled human serum albumin consisting of 0.5 to 1.5 μc per kilogram and a total of 1 to 5 mg. of albumin. The labeled preparations were obtained from Abbott Laboratories and contained less than 1 per cent nonprotein I^{131}. These lots were also used for tracer and blood volume studies in other individuals without proteinuria and had half-lives of 12 to 17 days with less than 1 per cent I^{131} urinary excretion in 4 hours. All dilutions of the stock I^{131}-labeled albumin solution were made with saline containing 0.5 per cent human serum albumin to serve as carrier and to reduce the probability of radiation effects on the labeled molecules. Lugol's solution, 10 drops 3 times a day, was given continuously starting 48 hours before the injection of the I^{131}-labeled albumin to block thyroid uptake of I^{131}.

Complete daily urine collections were obtained on all patients. Venous blood samples were drawn every one to two days without anticoagulant and the serum separated from the clot. For the purposes of this study, plasma and serum are used interchangeably. Total I^{131} activity was measured on 3 ml. aliquots of urine and serum. Nonprotein I^{131} in the urine was determined by precipitation of the protein in a 3 ml. aliquot with 3 ml. 20 per cent trichloroacetic acid for 24 hours at 5° C. and assay of I^{131} activity in 3 ml. of the supernatant after centrifugation. I^{131} albumin activity in the urine was calculated by subtracting the nonprotein from the total I^{131} activity. The same volume of I^{131} albumin solution in the same syringe used for the injection for each patient was diluted 1:5,000 with saline containing

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0 5 per cent albumin as carrier A 3 ml aliquot of this dilution was counted along with all samples as a standard to refer all calculations to the administered dose The use of this reference standard obviated the need to correct for I^{131} decay

Plasma volume was determined by the isotope dilution method, dividing the total activity injected by the activity in the serum 12 minutes after the injection The activity in the urine at this time was less than 0 1 per cent and could be neglected The 12 minute interval was selected as allowing adequate mixing in the circulation with minimum extravascular diffusion and metabolic loss

TABLE I SUMMARY OF PATIENTS STUDIED

GROUP AND PATIENT	SERUM			URINE	
	TOTAL PRO TEIN (GM %)	ALBUMIN (GM %)	CHOLESTEROL (MG %)	PROTEIN	DRB*
Group A Patients with latent nephrosis (little or no edema and minimal hypoalbuminemia)					
R T (BIH 30111), 30 year old white man, studied in October, 1955 Diagnosis chronic glomerulonephritis	5 1	3 3	407	3+	+
J C (BIH 70382), 35 year old white man, studied in October, 1955 Diagnosis chronic glomerulonephritis	5 4	3 4	400	4+	+
H G (BIH 65760), 30 year old white man, studied in June, 1957 Diagnosis diabetes mellitus	(October, 1956) 4 6	2 0	445	4+	+
	(June, 1957) 6 3	3 4	330	4+	+
Group B Patients with overt nephrosis (edema and moderate hypoalbuminemia)					
F S (BIH 65073), 29 year old white man, studied in March, 1956 Diagnosis subacute glomerulonephritis	4 3	1 4	340 400	4+	–
H L (BIH M 87292) 66 year old white woman, studied in June 1957 Diagnosis nephrotic syndrome of undetermined etiology	4 6	1 3	1 100 1 630	4+	+
M K (BIH M 85228), 50 year old white woman studied in June, 1957 Diagnosis nephrotic syndrome of undetermined etiology	4 3	2 1	520	4+	–

*Doubly refractile bodies

All counting was done with samples in test tubes placed in a well type sodium iodide crystal scintillation counter giving approximately 10^6 counts per minute per microcurie of I^{131}

Albumin in the urine and serum at the beginning, middle and end of the study was determined immunochemically using rabbit antiserum[4] and spectrophotometric analysis of the precipitates[5]

RESULTS

Plasma Disappearance of I^{131} Labeled Albumin —The plasma disappearance curves are shown in Fig 1 The interpretation of such curves has received considerable attention.[6, 7] The initial rapid decline over the first few days represents exchange of the injected I^{131} albumin with available albumin in the body as well as loss via the kidneys and catabolism. The subsequent slower decline over the remaining days to weeks represents disappearance due to the latter two factors. The constant rate of the slower decline on a semilogarithmic plot indicates that a constant fraction of the tracer remaining in the body each day is lost as a result of two processes—catabolism and excretion in the urine. Assuming the tracer behaves identically to endogenous albumin, then a similar constant fraction of the endogenous exchangeable pool is catabolized and lost via the kidneys each day.

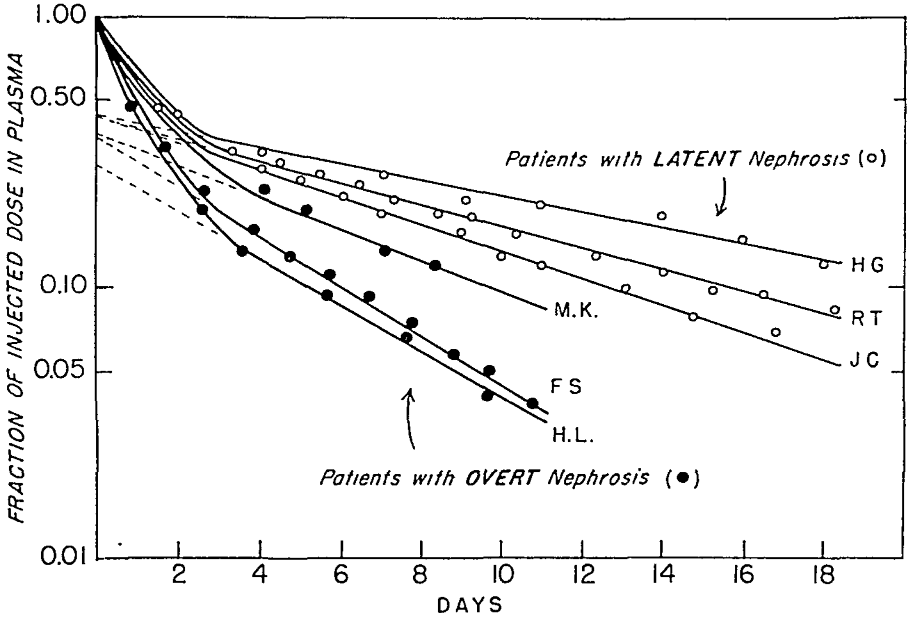

Fig. 1.—Plasma disappearance curves.

The results of graphic analysis of the plasma disappearance curves to determine the rates of disappearance of the tracer for these patients are shown in Table II. The normal range of turnover half-life for I^{131}-labeled albumin in adults is 10 to 17 days, corresponding to a turnover rate of 4 to 7 per cent of the body pool per day.[6-8] Since in the normal individual urinary loss is negligible, these figures represent rates of catabolism.

For Patients R. T. and J. C., who were edema-free, and H. G., with a trace of edema, these curves approach normal. The zero time intercept represents the fraction of the administered tracer that would have been present in the plasma had the tracer assumed its final volume of distribution initially. For these 3 patients, the intercepts are 0.42, 0.43, and 0.44; that is, the plasma I^{131} albumin would have been 42 to 44 per cent of the total I^{131} albumin if equilibrium had been obtained initially. This is in excellent agreement with the value obtained by others of 0.45 as the fraction of the total exchangeable albumin pool that is in the plasma.[6, 8]

TABLE II. I^{131} ALBUMIN TURNOVER DATA FROM PLASMA DISAPPEARANCE CURVES

PATIENT	ZERO TIME INTERCEPT	HALF LIFE (CATABOLISM PLUS URINARY LOSS) (DAYS)	RATE OF CATABOLISM PLUS URINARY LOSS* (PER CENT PER DAY)
Group A. Latent Nephrosis			
1. R. T.	0.43	7.3	9.5
2. J. C.	0.44	6.0	11.5
3. H. G.	0.42	10.5	6.6
Group B. Overt Nephrosis			
1. F. S.	0.38	3.0	23.0
2. H. L.	0.30	3.3	21.0
3. M. K.	0.39	5.0	13.9
Normal[6, 8]	0.45	10 to 17 days	4 to 7% per day (Catabolism)

$$\text{*Rate (per cent per day)} = \frac{0.693}{\text{half life}} \times 100$$

In contrast to these patients, the more rapid disappearance of the I^{131} labeled albumin from the plasma in the more severely nephrotic patients (F. S., H. L., and M. K.) is evident in Fig. 1. Since the disappearance curve represents the combined loss from albuminuria and catabolism it is not possible to determine their separate contributions from the plasma curves alone. The zero time intercepts of 0.30 and 0.38 in these nephrotic patients are much lower than the normal estimate of 0.45 as the fraction of the total albumin in the plasma, suggesting in nephrosis a higher than normal extravascular intravascular ratio of albumin. An excessive amount of extravascular albumin in nephrosis is unlikely since nephrotic edema fluid contains so little albumin.[3] The overestimation of extravascular albumin in nephrosis has been ascribed to the fact that the specific activity of the I^{131} albumin becomes higher in the edema fluid than in the plasma. This is presumably due to the slow return of the tracer to the circulation from edematous portions of the body. In Patient M. K. serial sampling of the edema fluid from the legs revealed that the specific activity of the edema fluid was twice the plasma specific activity after 5 days.

In edematous patients, therefore, the zero time intercept represents the final volume of distribution of the tracer and not of the endogenous albumin. Under these circumstances the plasma disappearance curves cannot be reliably used to calculate fractional rates of catabolism and urinary loss or to estimate the relative amounts of endogenous albumin in the vascular and extravascular compartments.

On the other hand, the urinary data provide a more reliable method of calculation for the rates of albuminuria, catabolism, and synthesis of albumin since the fraction of the amount of albumin synthesized per day which is lost in the urine is the same as the fraction of the administered tracer dose of I^{131} labeled albumin cumulatively recovered as I^{131} labeled albumin in the urine.[3] With patients in a steady state in relation to albumin metabolism the rate of albumin synthesis should equal the rate of albuminuria plus the rate of catabolism.

The rates of albuminuria, catabolism, and synthesis of albumin as calculated from the equations[3] are shown in Table III. A_u, the quantity of albumin in the urine per day, was determined immunologically at the beginning, middle, and end of the study, and these values were averaged. F_p', the fraction of the injected I^{131} activity appearing in the urine as I^{131} albumin, was obtained by subtracting the measured, cumulative nonprotein I^{131} activity from the total urinary I^{131} activity recovered as fractions of the administered dose. It is necessary to calculate *cumulative* recoveries since the daily clearance of nonprotein I^{131} iodide may be delayed with rapid degradation of I^{131}-labeled albumin, especially in the presence of renal disease.

TABLE III. RATES OF URINARY LOSS, CATABOLISM, AND SYNTHESIS OF ALBUMIN*

PATIENT	URINARY LOSS (A_U) (GM. PER DAY)	F_P'	SYNTHESIS (A_S) (GM. PER DAY)	CATABOLISM (A_C) (GM. PER DAY)
Group A. Latent Nephrosis				
1. R. T.	4.1	0.27	15.2	11.1
2. J. C.	7.1	0.40	17.8	10.7
3. H. G.	4.55	0.23	19.7	15.3
Group B. Overt Nephrosis				
1. F. S.	13.5	0.48	28.1	14.6
2. H. L.	5.8	0.66	8.9	3.1
3. M. K.	6.6	0.55	12.1	5.5

*$A_s = \frac{A_u}{F_p'}$ and $A_c = A_s - A_u$.

The absolute amounts of albumin synthesized, catabolized, or lost in the urine daily as shown in Table III are variable and not well correlated with the clinical state of the patient. In order to compare these various rates of albumin metabolism among the patients one must take into account the marked differences in the total amount of albumin in the body. To determine the *fractional* rates of catabolism and urinary loss, that is, the fractions of the albumin in the body catabolized and lost in the urine daily, requires an estimate of the size of the total body pool of albumin. The total pool of albumin has been estimated using the expression:

$$\text{total albumin pool} = \frac{\text{plasma pool}}{0.45},$$

the relationship in nonedematous individuals. The plasma pool is the plasma volume obtained by isotope dilution multiplied by the plasma albumin concentration. The fractional rate of catabolism or urinary loss is the absolute rate of catabolism or urinary loss divided by the estimated total albumin pool. These data are shown in Table IV.

From Table IV it is apparent that in only one patient, F. S., is there a marked increase in the fractional rate of catabolism of albumin. In the other patients, both with latent and overt nephrosis, the fractional rates of catabolism are within normal limits.

In the other edematous nephrotic patients, H. L., and M. K., the increased fractional rate of urinary loss appears to account for their severe albumin deficiency However, from Table IV it would appear that the absolute rates of synthesis of albumin, 8 9 and 12 1 Gm per day for Patients H L and M K, respectively, are far below the value of 28 1 Gm per day for Patient F S with a comparable degree of albumin deficiency In Patients H L and M K, therefore, impaired albumin synthesis may be a contributing factor to their albumin deficiency

TABLE IV ESTIMATED TOTAL ALBUMIN POOL AND FRACTIONAL RATES OF CATABOLISM AND URINARY LOSS OF ALBUMIN

PATIENT	PLASMA VOLUME (LITERS)	PLASMA ALBUMIN (GM / 100 ML)	PLASMA POOL (GM)	TOTAL POOL (GM)	FRACTIONAL RATE OF CATABOLISM (FRACTION PER DAY)	FRACTIONAL RATE OF URINARY LOSS (FRACTION PER DAY)
Group A	*Latent Nephrosis*					
1 R T	2 32	3 63	85 1	189	0 059	0 022
2 J C	3 01	3 32	100	222	0 048	0 032
3 H G	2 95	3 40	100	222	0 069	0 020
Group B	*Overt Nephrosis*					
1 F S	2 84	1 45	41 2	91 5	0 160	0 148
2 H L	2 98	1 09	32 5	72 4	0 043	0 081
3 M K	2 21	2 00	44 2	98 1	0 056	0 067
Normal	3 0	4 0	120	267	0 04 to 0 07	0 0004

These data indicate that the adult patients with *latent* nephrosis do not have a metabolic abnormality of albumin metabolism in that the fractional rate of catabolism is normal and the amount of albumin synthesized daily is sufficient *to compensate for the urinary loss with little, if any, reduction in the* total pool of albumin In contrast, one of the patients with recent *overt* nephrosis and mild edema during the study has a markedly increased fractional rate of catabolism contributing as much to the increased albumin turnover and albumin deficiency as the massive urinary loss In the other two patients with *overt nephrosis, the fractional rate of catabolism is normal and urinary loss is* considerable, albumin synthesis in these patients appears insufficient to overcome the urinary loss, resulting in a marked reduction in the total body pool of albumin

DISCUSSION

Since this study involves assumptions concerning the validity of I^{131} labeled albumin as a tracer for endogenous albumin, some data bearing on this problem will be reviewed Turnover studies in human beings have been reported[9 12] using albumin labeled with I^{131}, C^{14}, and S^{35}, and the rates of degradation of albumin have not always agreed by these methods The S^{35} and C^{14} labels introduce the problem of reincorporation of the degraded activity whereas I^{131} degraded from albumin appears entirely as nonprotein iodide in the urine when thyroid uptake is blocked A crucial factor in determining the turnover of I^{131} labeled albumin is the degree of iodination Excessive iodination, above a few atoms of iodine per molecule of albumin, leads to a more rapid degradation[7] Denaturation resulting in an increased rate of degradation may occur from self-radiation[13]

Despite these problems, if the same or similar preparations of I^{131}-labeled albumin as were used were catabolized somewhat faster than endogenous albumin, there still would be a fixed relation between I^{131}-labeled and endogenous albumin metabolism. The differences found among the patients in this study would remain real, although the quantitative estimates of the various rates of metabolism might be in error.

The patients were assumed to be in a steady state as regards albumin metabolism so that the rate of albumin synthesis would remain equal to the rate of catabolism plus urinary loss throughout the study period. The criteria upon which this assumption is based were the negligible (less than 10 per cent) changes in serum albumin concentrations, urinary albumin, and body weight and an unchanged clinical status during the study.

Trapping of I^{131}-labeled albumin may lead to a higher specific activity of albumin in edema fluid compared to plasma. The extrapolated value for the ratio of plasma albumin to total exchangeable albumin from the plasma die-away curve would be too low under these circumstances. For the calculation of fractional rates in the patients with overt nephrosis the value of 0.45 as the ratio of plasma albumin to total albumin found in normal individuals was used instead of the observed lower values of 0.30 to 0.38. Even if the lowest value of 0.30 were used for Patient F. S., his total albumin pool would be increased from 91.5 to 137 Gm. and the fractional rate of catabolism would drop from 0.16 to 0.11. This would still represent a markedly accelerated fractional rate of catabolism compared to the normal range of 0.04 to 0.07.

The problem of unequal specific activities in edematous patients receiving I^{131}-labeled albumin has been reviewed by Kushner and associates[14] who found a threefold higher specific activity of edema fluid albumin compared to plasma in a patient with nutritional anasarca. Equal specific activities were found in 2 other edematous patients, 1 with nephrosis. In the one patient whose edema fluid was sampled in this study (Patient M. K.), the specific activity of the edema fluid albumin was twice that of the plasma after 5 days. One of the factors that may account for the discrepancy in specific activities among investigators is the method used for the determination of albumin when present in small quantities. Hypoproteinemic edema fluid of the type seen in nephrosis may have albumin concentrations as low as 10 mg. per cent. This amount can be accurately determined by the immunologic method using rabbit antiserum but concentration techniques and electrophoresis may lead to an overestimation of the albumin content and hence a lower specific activity.

In contrast to the findings in a study of children with the nephrotic syndrome,[3] in whom excessive catabolism was uniformly found with severe albumin depletion and anasarca, only 1 of the 3 adult nephrotic patients with fairly severe hypoalbuminemia had an excessive fractional rate of catabolism. However, these adult patients showed neither as much edema nor as severe albumin deficiency as the children classified as severe cases with marked anasarca and serum albumin levels of 0.5 Gm. per 100 ml. or less, in whom catabolism and urinary loss of albumin were both excessive. The adults with overt nephrosis in this study are more comparable clinically to the children (in Group II) with *moderate* albumin depletion and some edema, in whom excessive catabolism was not uniformly observed. It is interesting that the adult patient in this study with the increased fractional rate of catabolism of albumin was much younger than the other 2 overtly nephrotic patients with normal fractional rates of catabolism. In the study of children with nephrosis, albumin synthesis was always in the high normal range; in contrast, among these adults impaired synthesis of albumin may have been present in the 2 oldest with overt nephrosis. In these patients, H. L. and M. K. (66 and 50 years old respectively), the usual liver function tests were normal. M. K. received Meticorten during the study; however, it is unlikely that steroid therapy significantly influenced the results since the patient showed no clinical change and levels of albumin in the serum and urine were the same before, during, and after steroid therapy.

In an earlier study of I^{131} labeled albumin in nephrotic adults Blahd and co-workers[15] interpreted the results as indicating no abnormality of catabolism. Their methods and calculations were not comparable to the present study, and it is not clear how rates of albumin synthesis and catabolism could be determined from a metabolic nitrogen balance and plasma I^{131} albumin die-away plots alone.

The mechanism of excessive catabolism of albumin in the nephrotic syndrome, when present, is not known. Abnormal catabolism is independent of the total amount of albumin in the body, since the fraction of the albumin pool catabolized by Patients M. K. and H. L. was normal, yet for Patient F. S. with a comparable total pool the fraction catabolized was far above normal. The kidney has been implicated as a possible site of the normal degradation of proteins.[16] The role of the kidney in the excessive catabolism of albumin found in some nephrotic patients has not yet been established.

SUMMARY

1. I^{131} labeled albumin turnover studies were carried out in 3 adult patients with latent nephrosis and 3 with overt nephrosis. One of the 6 adults, a 30 year old man with hypoalbuminemia and mild edema, showed a striking increase in the fractional rate of catabolism of albumin which contributed as much to the depletion of the body pool of albumin as did the massive urinary loss. In this patient the rate of albumin synthesis was increased.

2. Some impairment of albumin synthesis may have been a contributing factor to the albumin deficiency in the 2 other cases of overt nephrosis in whom the rate of catabolism of albumin was normal. These 2 patients were 50 and 66 years old, respectively.

3. Rates of albumin catabolism and synthesis were normal in the 3 patients with latent nephrosis.

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CHANGES IN BODY COMPOSITION DURING THE COURSE OF ACUTE ANTERIOR POLIOMYELITIS

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THE important role potassium plays in various physiologic processes has been documented in several recent reviews[1 6], however, relatively few studies of potassium metabolism during the course of acute anterior poliomyelitis had been reported prior to the initiation of our investigation, and the conclusions drawn from the observed data were contradictory Earle[7] found no correlation between serum potassium and severity of paralysis and the administration of potassium chloride to his paralyzed patients did not appear to improve muscle function In contrast, Lans and associates[8] reported that the administration of potassium chloride to 3 patients suffering from bulbar poliomyelitis with hypokalemia produced marked improvement in their clinical condition Bower and his colleagues[9, 10] measured serum potassium concentration and the excretion of potassium in the urine during the course of acute anterior poliomyelitis They found the excretion of potassium in the urine to be consistently increased, while the serum potassium was elevated if the patients were "dehydrated" and/or oliguric, and normal or decreased if the patients "were adequately hydrated " They concluded an intracellular deficit of potassium developed during the course of acute anterior poliomyelitis and this deficit was indicated by the presence of hypokalemia, provided the patient was adequately hydrated and was not oliguric In view of these conflicting conclusions we elected to study serially the intracellular potassium content, as defined by isotopic dilution techniques,[11] of patients suffering from acute anterior poliomyelitis

METHODS

Subjects—The 45 subjects described in this report were selected from the group of patients hospitalized in the Municipal Contagious Disease Hospital (MCDH) during the 1956 poliomyelitis epidemic in Chicago This patient population comprised approximately 75 per cent of the individuals who had a diagnosis of acute anterior poliomyelitis reported to the city health authorities during the epidemic Tables I and II include pertinent clinical data Age was recorded to the nearest birthday Onset of illness was measured from the date of the initial symptom recorded in the chart by the examining physician at MCDH Because evaluation of muscle strength is difficult during the acute illness, the Lovett method for evaluating paralysis was simplified for the purposes of this study in the following manner 3 plus, at

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most, complete range of motion with gravity eliminated; 2 plus, complete range of motion against gravity with minimal resistance; plus, complete range of motion against gravity with moderate resistance; minus, complete range of motion against gravity with full resistance. Severity of illness was arbitrarily evaluated by giving one plus point for each of the following factors: a day of fever, paralysis in each extremity for each extremity, paralysis of the trunk, paralysis of cranial nerves, catheterization of the bladder, tracheotomy, nasopharyngeal suction, and each complication. Methods of treatment have been previously described.[12] It was not possible to evaluate fluid or electrolyte balance during the course of the disease; however, in acutely ill patients the daily water and electrolyte intake was limited to 500 ml. of 5 per cent dextrose in water, 100 ml. of isotonic sodium chloride, and 3.0 Gm. of potassium chloride. Sodium and potassium salts were not administered during the days of the study. Patients not in respirators were weighed nude on a cart scale to an accuracy of ±0.1 Kg. It was not possible to perform serial measurements on all of the patients admitted to the study because the patients who did not develop complications were discharged after the acute phase of their illness passed. The study was limited to adults and large adolescents for the following reasons: (1) older patients are more likely to develop severe illness and, therefore, would be more apt to show changes in intracellular potassium content, if any occur, and (2) the need for generous amounts of blood for analysis. All of the patients studied serially were critically ill and the majority of them required continuous care in a tank respirator. None of the subjects were pregnant.

Procedure.—Studies were initiated twice a week, usually on Monday and Wednesday. Patients were incorporated into the investigation shortly after their admission to the hospital. Serial studies, when performed, were done at 7- to 10-day intervals. Approximately 200 μc of K^{42} as potassium carbonate* was injected in a potassium and sodium chloride solution, in a quantitative manner previously described[13] between 9:30 and 10:30 A.M. of the first day. All urine subsequently voided was collected and the collection period closed just prior to the quantitative injection of radiosulfate† and antipyrine‡ between 7:30 and 8:30 A.M. of the following day. Urine was quantitatively collected 1½, 3, 4½, and 6 hours after the injection of radiosulfate and antipyrine for the determination of total exchangeable potassium and radiosulfate space. Blood samples were obtained 3, 4, 5, and 6 hours after injection of radiosulfate and antipyrine for the determination of radiosulfate and antipyrine spaces. The methods for determination of radiosulfate space and antipyrine space have been reported.[14] In an appreciable number of patients, extrapolation of the radiosulfate counts excreted in the urine to zero time gave a number larger than the actual counts injected. When this occurred actual counts injected were used to compute the radiosulfate space. Serum and urine samples were frozen and stored until analyses of antipyrine and assay of sulfate radioactivity were to be done.

Total exchangeable potassium was computed by the following formula:

$$K_e = \frac{\text{counts } K^{42} \text{ injected} - \text{counts } K^{42} \text{ excreted in urine}}{\text{specific activity of urine sample}}.$$

Radioactivity of K^{42} was assayed in duplicate in a well-type scintillation counter to a counting error less than 1 per cent. Radiosulfate activity was not detected by the scintillation counter. Quadruplicate assay of standards of K^{42} and of radiosulfate was done in all cases. Standards were prepared by diluting the same volume (5 ml.) injected into patients to produce a counting rate approximating that observed in the urine of the subjects. Potassium was measured by a flame photometer. The mean K_e was determined by averaging the K_e obtained from the 4 urine samples. The coefficient of variation for the 69 determinations of total exchangeable potassium was $\overline{C} = 1.86 \pm 1.53$ per cent. The coefficient of variation of the 242 duplicate chemical determinations of antipyrine was $C = 0.473 \pm 0.513$ per cent. In 16 samples, sufficient serum was not available for duplicate analysis for antipyrine. The following symbols and abbreviations have been employed:

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*Prepared by irradiation in the reactor at the Argonne National Laboratories, Lemont, Ill., on allocation from the AEC.

†Obtained from Abbott Laboratories, North Chicago, Ill., by license from the AEC.

‡Generously supplied by Dr. B. L. Martz, Eli Lilly & Company, Indianapolis, Ind.

$(K)_s$ = Serum potassium concentration
K_e = Total exchangeable potassium
TBW = Total body water (antipyrine space)
ECF = Extracellular fluid volume (radiosulfate space)
ICF = Intracellular fluid volume = TBW − ECF
LBM = Lean body mass = TBW/0.73
B Wt = Body weight
K_{LBM} = Potassium content of lean body mass = K_e/LBM
ECFK = Extracellular content of potassium = ECF × $(K)_s$
ICFK = Intracellular content of potassium = K_e − ECFK
K_{ICF} = Intracellular water concentration of potassium = ICFK/ICF
K_e/B Wt = Total exchangeable potassium per kilogram of body weight
μ = Mean (bar over variable denotes mean)
σ = Standard deviation using N 1
M = Male
F = Female
M_A = Initial studies of all males
F_A = Initial studies of all females
F_N and M_N = Serial studies where N denotes the number of the study
C = Coefficient of variation
r = Coefficient of correlation
t = time

RESULTS

The measured and derived data for males are presented in Table I and for females in Table II. A summary of the means ± 1 standard deviation of selected measured and derived variables is presented in Table III.

Clinical Data.—There were no significant differences ($P > 0.05$) between the male and female patients with respect to age, the time after onset of the disease the studies were done, and the severity of illness. The differences in distribution by race within each sex were not significant by the χ^2 test ($P > 0.05$).

Serum Potassium.—$[\overline{K}]_s$ was within normal limits for all groups. There were no significant differences ($P > 0.05$) between $[\overline{K}]_s$ of corresponding subgroups of males and females. There were no significant differences ($P > 0.05$) between $[\overline{K}]_s$ of each of the subgroups of males compared to each other or of each of the subgroups of females compared to each other. In addition there was no significant ($P > 0.05$) correlation between $[K]_s$ and K_{ICW} or K_{LBM}.

Total Exchangeable Potassium.—Fig. 1 shows the regression of K_e on LBM for all of the initial studies of the males and females, respectively. Statistical analysis of the slope of each line indicates there is a significant regression ($P < 0.001$) of K_e on LBM. Statistical analysis of the intercept of each line indicates that the intercept does not significantly ($P > 0.05$) differ from zero

TABLE I. CLINICAL, EXPERIMENTAL, AND DERIVED DATA OF MALE SUBJECTS

PATIENT	AGE	RACE	SEVERITY OF ILLNESS*	B. WT. (KG.)	DAY AFTER ONSET†	SERUM (K) (mEq./L.)	K_e (mEq.)	TBW		ECF		ICF (L.)	K_{ICF} (mEq./L.)	LBM (KG.)	K_{LBM} (mEq./KG.)	ECF_{TBW}	ICF_{TBW}	ECF_{ICF}	K_e/B. WT. (mEq./KG.)
								L.	% B. WT.	L.	% B. WT.								
K. P.	13	W	4	56.4	14	3.65	2385	37.3	66.1	12.2	21.6	25.1	93.2	51.1	46.7	0.33	0.67	0.49	42.3
D. G.	19	N	36	75‡	17	4.15	2851	32.2	–	15.0	–	17.2	162	44.1	64.6	0.47	0.53	0.87	–
–				–	27	4.60	2310	34.0	–	14.2	–	19.8	113	46.6	49.6	0.42	0.58	0.72	–
–				–	33	4.30	2304	33.2	–	16.1	–	17.1	131	45.5	50.6	0.49	0.51	0.94	–
E. St.	21	W	44	98‡	7	4.42	4110	39.6	–	20.0	–	19.6	205	54.3	75.7	0.51	0.49	1.06	–
–				–	14	4.08	3266	45.8	–	33.9	–	11.9	263	62.7	52.1	0.74	0.26	2.90	–
–				–	25	4.90	2285	37.5	–	19.3	–	18.2	120	51.4	44.5	0.51	0.49	1.10	–
J. K.	14	W	3	65.8	7	5.25	2313	25.7	39.1	10.1	15.4	15.6	145	35.2	65.7	0.39	0.61	0.65	35.1
H. P.	27	N	27	55‡	10	3.40	2035	25.2	–	6.5	–	18.7	108	34.5	59.0	0.26	0.74	0.35	–
–				–	19	3.75	1859	28.0	–	6.9	–	27.1	87	38.4	48.4	0.25	0.75	0.33	–
G. E.	17	W	1	67.1	7	3.70	3237	38.2	57.0	12.5	18.7	25.7	124	52.3	61.9	0.33	0.67	0.49	48.2
H. S.	25	W	9	78.1	7	4.82	3580	39.4	50.5	13.5	17.3	25.9	136	54.0	66.3	0.34	0.66	0.52	45.8
C. L.	22	N	15	–	12	5.00	3161	30.8	–	11.0	–	19.8	157	42.2	74.9	0.36	0.64	0.56	–
W. G.	34	N	14	89.5	4	3.40	4644	42.0	46.9	14.7	16.4	27.3	168	57.5	80.8	0.35	0.65	0.54	51.9

T M	28	N	30	68*	5	3 95	2493	31 2	–	12 2	–	19 0	129	42 7	58 4	0 39	0 61	0 64	–
–				–	19	4 10	2208	31 8	–	11 7	–	20 1	108	43 6	50 6	0 37	0 63	0 58	–
J P	13	W	31	–	7	4 05	1647	21 4	–	6 2	–	15 2	107	29 3	56 2	0 29	0 71	0 41	–
–				–	16	3 00	1293	24 0	–	8 0	–	16 0	79	32 9	39 3	0 33	0 67	0 50	–
–				–	28	2 90	967	17 3	–	6 8	–	10 5	90	23 7	40 8	0 39	0 61	0 65	–
–				30	37	4 52	1126	19 2	64 0	8 7	29 0	10 5	104	26 3	42 8	0 45	0 55	0 83	37 5
J L	23	W	6	62 8	4	3 60	3169	27 5	43 8	10 6	16 8	16 9	185	37 7	84 1	0 39	0 61	0 63	50 5
J Ro	24	W	7	68 1	5	3 65	2569	34 0	50 0	9 5	13 9	24 5	103	46 6	55 1	0 28	0 72	0 39	37 7
A G	21	W	2	59 0	9	3 65	3005	31 9	54 0	12 2	20 7	19 7	150	43 7	68 8	0 38	0 62	0 62	50 9
J E	20	N	3	64 2	5	3 88	3883	34 1	53 1	11 2	17 5	22 9	168	46 7	83 2	0 33	0 67	0 49	60 5
H B	26	W	6	62 5	6	3 82	3025	31 1	49 7	11 4	18 2	19 7	151	42 6	71 0	0 37	0 63	0 58	48 4
Em W	21	N	17	54 4	6	3 65	2877	36 4	67 0	11 1	20 4	25 3	112	49 9	57 7	0 31	0 69	0 44	52 9
J Ra	29	N	15	–	8	3 86	3323	32 3	–	11 6	–	20 7	158	44 3	75 0	0 36	0 64	0 56	–
–				65 1	15	4 18	2566	35 0	53 8	12 1	18 7	22 9	110	47 9	53 6	0 35	0 65	0 53	39 4
M M	32	N	8	75 1	6	3 60	3051	35 7	47 5	13 2	17 6	22 5	134	48 9	62 4	0 37	0 63	0 59	40 6
L D	28	W	0	84 5	7	3 95	3447	40 5	47 9	14 4	17 0	26 1	130	55 5	62 1	0 36	0 64	0 55	40 8

*See text for method of computation

†From time of onset to initial study

‡Last known body weight

TABLE II. CLINICAL, EXPERIMENTAL, AND DERIVED DATA OF FEMALE SUBJECTS

PATIENT	AGE	RACE	SEVERITY OF ILLNESS*	B. WT. (KG.)	DAY AFTER ONSET†	SERUM (K) (mEq./L.)	K_e (mEq.)	TBW		ECF		ICF (L.)	K_{ICF} (mEq./L.)	LBM (KG.)	K_{LBM} (mEq./KG.)	ECF_{TBW}	ICF_{TBW}	ECF_{ICF}	K_e/B. WT. (mEq./KG.)
								L.	% B. WT.	L.	% B. WT.								
M. P.	18	W	26	64‡	14	–	1901	–	–	–	–	–	–	–	–	–	–	–	–
D. B.	26	W	11	78.3	7	–	1528	–	–	–	–	–	–	–	–	–	–	–	19.5
–				77.2	24	4.25	1573	23.6	30.5	7.9	10.3	15.7	98.1	32.3	48.7	0.33	0.67	0.50	20.4
K. G.	9	W	14	40.1	17	4.42	1272	19.5	48.6	8.3	20.8	11.2	110	26.2	48.5	0.43	0.57	0.74	31.7
–				39.2	31	5.40	1311	17.8	45.5	8.0	20.3	9.8	129	24.4	53.7	0.45	0.55	0.82	33.4
C. F.	14	W	8	35.9	28	4.18	1362	19.3	53.9	6.8	18.9	12.5	107	26.4	51.6	0.35	0.65	0.54	37.9
M. He.	12	N	3	32.7	10	5.40	1315	19.1	58.5	6.6	20.2	12.5	102	26.2	50.2	0.35	0.65	0.53	40.2
R. Z.	33	W	30	45‡	13	3.63	969	16.7	–	6.4	–	10.3	93	22.9	42.3	0.38	0.62	0.62	–
–				–	23	4.10	1130	17.7	–	11.1	–	6.6	164	24.3	46.5	0.63	0.37	1.68	–
–				–	29	4.78	1067	15.6	–	8.5	–	7.1	145	21.4	49.9	0.55	0.45	1.20	–
M. Ha.	28	W	16	50.6	11	4.27	1810	22.1	43.6	12.5	24.7	9.6	183	30.3	59.7	0.57	0.43	0.30	35.8
L. C.	12	N	13	36	17	4.42	1651	20.9	58.0	6.3	17.6	14.6	111	28.6	57.7	0.30	0.70	0.43	45.9
H. C.	23	W	4	67.6	6	4.25	1829	23.9	35.3	8.6	12.7	15.3	117	32.7	55.9	0.36	0.64	0.56	27.1
J. B.	27	W	10	49.8	12	3.75	1974	25.6	51.4	15.8	31.8	9.8	195	35.1	56.2	0.62	0.38	1.61	39.6
E. So.	40	W	10	91.0	9	3.70	2399	32.8	36.0	10.8	11.9	22.0	107	44.9	53.4	0.33	0.67	0.49	26.4
M. F.	22	W	0	71.3	5	4.00	2149	29.3	41.1	9.6	13.4	19.7	107	40.1	53.6	0.33	0.67	0.49	30.1
B. G.	26	W	8	50.7	7	3.95	1859	25.8	50.9	9.1	18.0	16.7	109	35.3	52.7	0.35	0.65	0.55	36.7
B. V.	25	W	9	61.5	21	4.48	1615	24.3	39.5	8.2	13.3	16.1	98	33.3	48.5	0.34	0.66	0.51	26.3
E. H.	23	W	3	54.0	11	4.48	1933	25.0	46.2	9.0	16.7	16.0	118	34.3	56.4	0.36	0.64	0.56	35.8
M. B.	24	W	10	51.5	7	3.28	2085	25.8	50.2	7.8	15.1	18.0	114	35.3	59.1	0.30	0.70	0.43	40.5
M. J.	27	W	39	–	13	3.18	1330	25.4	–	10.1	–	15.3	85	34.8	38.2	0.40	0.60	0.66	–
–				–	20	3.40	1394	23.8	–	11.2	–	12.6	108	32.6	42.8	0.47	0.53	0.89	–
–				–	34	4.10	1174	20.1	–	8.8	–	11.3	101	27.5	42.7	0.44	0.56	0.78	–

L S	13	W	36	–	6	3.48	1667	24.1	–	9.4	–	14.7	111	34.0	50.5	0.39	0.61	0.64	–
–				–	13	4.25	1490	20.3	–	7.1	–	13.2	111	27.8	51.6	0.35	0.65	0.54	–
–				41.4	20	4.66	1143	18.3	44.2	9.4	22.7	8.9	124	25.4	45.5	0.51	0.49	1.06	27.6
B A	24	W	40	54‡	5	3.86	1797	23.7	–	10.2	–	13.5	130	32.5	55.3	0.43	0.57	0.76	–
–				–	12	3.65	1569	23.0	–	9.0		14.0	110	31.5	49.8	0.39	0.61	0.64	–
–				–	24	4.18	1332	19.4	–	8.4	–	11.0	118	26.0	50.1	0.43	0.57	0.76	–
F I W	28	N	56	–	8	4.23	2027	26.4	–	9.4	–	17.0	117	36.2	56.0	0.36	0.64	0.55	–
–				–	15	3.82	1845	23.9	–	9.9	–	14.0	129	32.7	56.4	0.41	0.59	0.71	–
–				–	27	3.52	1478	24.7	–	13.0	–	11.7	122	33.8	43.7	0.53	0.47	1.11	–
G M	31	N	6	77.4	10	4.90	2547	26.5	34.2	9.8	12.7	16.7	150	36.3	70.2	0.37	0.63	0.59	32.9
A I	18	N	4	52.8	4	4.10	1947	24.5	46.4	7.5	14.1	17.0	113	34.6	57.9	0.31	0.69	0.44	36.9
R S	24	W	26	–	5	3.82	2380	27.6	–	7.4	–	20.2	116	37.8	63.0	0.27	0.73	0.37	–
–				–	12	4.70	1823	29.4		14.1	–	15.3	115	40.3	45.2	0.48	0.52	0.92	–
				–	19	4.00	1806	27.6	–	10.7		16.9	104	37.8	47.8	0.39	0.61	0.63	–
P I	26	W	21	–	12	3.40	1165	20.6	–	10.5	–	10.1	112	28.2	41.3	0.51	0.49	1.04	–
B P	21	W	2	48.0	6	4.10	1855	27.4	57.1	10.7	22.4	16.7	109	37.5	49.5	0.39	0.61	0.64	39.6

*See text for method of computation

†From time of onset to initial study

‡Last known body weight

TABLE III. SUMMARY OF $\mu \pm \sigma$ OF SELECTED EXPERIMENTAL AND DERIVED DATA

	INITIAL STUDIES	SERIAL STUDIES				
MALES	ALL SUBJECTS (20)*	1 (6)*	2 (6)*	3 (3)*	4 (1)*	MIXED
$[K]_S$ (mEq./L.)	3.97 ± 0.52	3.97 ± 0.31	3.95 ± 0.54	4.03 ± 1.04	4.52	–
K_{LBM} (mEq./Kg.)	66.5 ± 10.2	64.8 ± 8.7	48.9 ± 5.1	45.3 ± 5.0	42.8	–
K_{ICW} (mEq./Kg.)	141 ± 29.3	145 ± 37.8	127 ± 68.2	114 ± 21.2	104	–
K_t/B. Wt. (mEq./Kg.)	46.6 ± 7.1†	–	–	–	37.5	38.5 ± 1.3‡
ECF_{TBW}	0.36 ± 0.059	0.38 ± 0.098	0.41 ± 0.17	0.46 ± 0.07	0.45	–
ICF_{TBW}	0.64 ± 0.059	0.62 ± 0.098	0.59 ± 0.17	0.54 ± 0.07	0.55	–
ECF_{ICF}	0.57 ± 0.16	0.64 ± 0.26	0.93 ± 0.97	0.90 ± 0.23	0.83	–
Day after onset study done	7.7 ± 3.4	9 ± 4.2	18 ± 4.7	29 ± 3.5	37	–
Severity of illness	13.9 ± 13	30.5 ± 9.6	–	–	–	–
FEMALES	ALL (23)*	1 (7)*	2 (8)*	3 (6)*	–	–
$[K]_S$ (mEq./L.)	4.06 ± 0.52	3.80 ± 0.41	4.20 ± 0.63	4.21 ± 0.46	–	–
K_{LBM} (mEq./Kg.)	53.4 ± 7.7	50.5 ± 8.5	49.6 ± 4.7	46.6 ± 3.1	–	–
K_{ICW} (mEq./Kg.)	118 ± 24.7	109 ± 15.2	120.5 ± 20.5	119 ± 15.9	–	–
K_e/B. Wt. (mEq./Kg.)	34.2 ± 6.7§	–	–	–	–	27.1 ± 6.5‖
ECF_{TBW}	0.38 ± 0.084	0.38 ± 0.055	0.44 ± 0.094	0.47 ± 0.064	–	–
ICF_{TBW}	0.62 ± 0.084	0.62 ± 0.055	0.56 ± 0.094	0.53 ± 0.064	–	–
ECF_{ICF}	0.65 ± 0.29	0.62 ± 0.13	0.84 ± 0.37	0.92 ± 0.23	–	–
Day after onset study done	10.6 ± 6.0	9.6 ± 4.8	18 ± 7.1	25.5 ± 5.7	–	–
Severity of illness	16.0 ± 15	34.4 ± 13.1	–	–	–	–

*Parentheses indicate number of patients in study.

†Thirteen patients.

‡Two patients

§Seventeen patients

‖Three patients

$\overline{K}_e$ decreased during the course of the illness, however, the decrease was not significant ($P > 0.05$). The line of regression of K_e on LBM was derived for each serial study of males and females. Statistical analysis of each line indicated that there was a significant regression ($P < 0.05$) of K_e on LBM for each line and the intercept of each line did not differ significantly from zero ($P > 0.05$).

LBM Content of Potassium (Fig. 2).—The initial $\overline{K}_{LBM}$ for all males was 66.5 mEq./Kg. and for all females 53.4 mEq./Kg. The difference is significant ($P < 0.001$). The difference between males and females is significant for the initial serial studies ($P < 0.025$). The males showed a significant decrease in K_{LBM} during the course of the illness as indicated in Table IV. A significant decrease ($P < 0.05$) in K_{LBM} of females was present only between the initial study of all of the female patients and the third serial study of the female patients. When the subjects were classified by race the pattern was the same, however, *the differences between males and females were significant only for* the white subjects ($P < 0.001$).

TABLE IV. PROBABILITY VALUES OBTAINED BY COMPARING $\overline{K}_{LBM}$ OF MALES AND $\overline{K}_{LBM}$ OF EACH SUBGROUP OF MALES

SUBJECTS COMPARED	P
$M_A - M_1$	> 0.05
$M_A - M_2$	< 0.001
$M_A - M_3$	< 0.005
$M_1 - M_2$	< 0.005
$M_1 - M_3$	< 0.01
$M_2 - M_3$	> 0.05

Intracellular Water Potassium Concentration.—The initial $\overline{K}_{ICF}$ for all males was 145 mEq./L. and for all females 118 mEq./L. This difference is significant ($P < 0.01$). A significant difference ($P < 0.05$) was also demonstrated between the $\overline{K}_{ICF}$ of males and of females in the first serial study. Other differences are not significant ($P > 0.05$). During the course of the illness K_{ICF} for males decreased, however, the decrease was not significant statistically ($P > 0.05$). There was little change in $\overline{K}_{ICF}$ for females during the course of the illness.

Exchangeable Potassium Per Kilogram of Body Weight.—The initial $\overline{K}_e$/B. Wt. for males was 46.6 mEq./Kg. and for females 34.2 mEq./Kg. The difference is significant ($P < 0.001$). K_e/B. Wt. could be computed for a few patients studied serially and decreased during the course of the illness. The difference between males and females in the repeat studies is significant ($P < 0.05$).

Body Water.—Between the initial and second study TBW increased 9.1 per cent in males and decreased 4.4 per cent in females. This difference is significant ($P < 0.05$). By the third serial study TBW had decreased below the initial value in males and there was an additional decrease in the females. There is no significant difference in the distribution of water between the extracellular

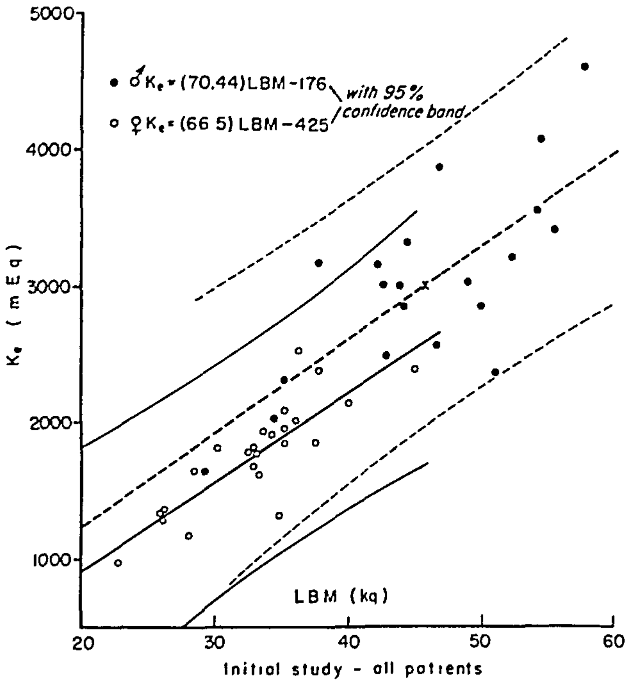
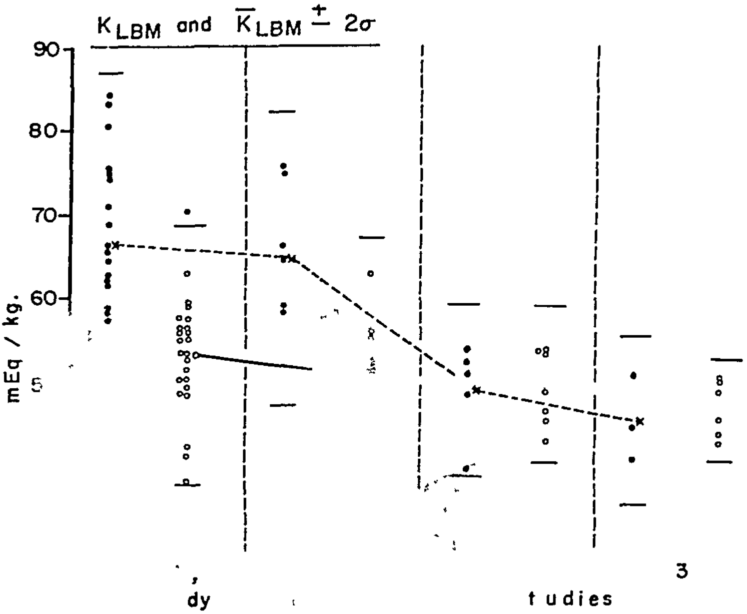


Fig. 1.—Regression of total exchangeable potassium upon lean body mass.



2 —T potassium.

and intracellular water compartments when the mean values for corresponding subgroups of males and females are compared ($P > 0.05$). During the course of the illness there is a change in the distribution of water, ECF is increased and ICF is decreased, this change is significant in males ($P < 0.005$) and females ($P < 0.05$).

Single Serial Study (Fig. 3).—One of the male patients (postpubertal) studied serially had considerable recovery of muscle function and was able to leave the respirator. It is of interest to note that his clinical course was associated with hypokalemia, decreased K_{ICW}, and a decreased K_{LBM}. All of these increased toward values found in clinically healthy subjects (see Discussion), with clinical improvement. The changes in body water distribution were characteristic of those observed for the group as a whole. K_e decreased during the illness and increased with recovery.

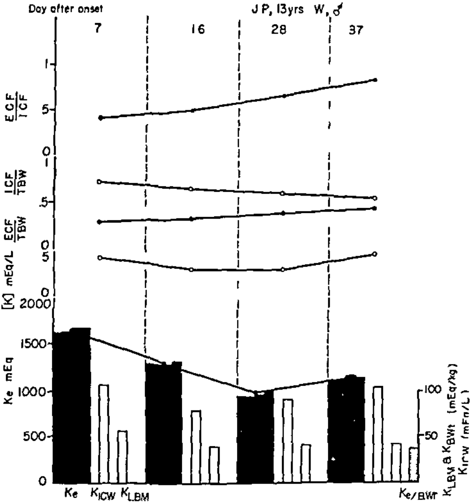


Fig. 3.—Changes in body composition obtained by serial study of Patient J. P.

DISCUSSION

We have elected to employ the lean body mass model of Behnke[15] as modified by Pace and his co-workers[16-18] for reasons that will be evident later in the discussion. Keys and Brozek[19] have two criticisms of the lean body mass model (1) the inclusion of the indefinite concept of essential lipids into the model and (2) the belief that the composition of the lean body mass model is not independent of the total fat content of the body. However, the concept of essential lipids does not affect our use of the LBM model and their belief that body fat significantly affects the composition of the lean body mass is based on computational errors. They give correlation coefficients on page 282 of reference 19, computed from the data of Pace and his colleagues[16-18] which, they state, show a significant correlation between total body fat and percentage of water in the fat-free body. Our own computations from these data give $r_M = 0.3082$ and $r_F = 0.3575$; these correlation coefficients are not significant. Since the intercept of each of the regression lines does not differ significantly from zero, we can replace the line defined by the equation $K_e = b$ (LBM) + constant, by the line $K_e = b$ (LBM), compute b for each group and compare the results. Evidence supporting this extrapolation of the regression lines will be cited later in the discussion. Granting the previous assumptions, it is evident that we have demonstrated a significant difference in potassium metabolism between males and females during the course of acute anterior poliomyelitis. In females there is little change in K_{ICW} and a significant (when F_3 is compared to F_A) decrease in K_{LBM} during the course of the illness. In males, K_{ICW} and K_{LBM} decrease during the course of the illness; however, the decrease is significant only for K_{LBM}. By the third serial study, K_{LBM} and K_{ICW} are not significantly different for males and females. The changes for K_{ICW} are presumably not as clear-cut as for K_{LBM} because of the larger experimental error introduced into the calculation of K_{ICW} in accordance with the law of the propagation of errors. That these differences are not secondary to changes in (1) extracellular content or concentration of potassium is suggested by the lack of any significant correlation of ECFK or $[K]_s$ with K_{ICW} or K_{LBM}, or (2) water metabolism is suggested by the occurrence in males and females of identical changes in the distribution of water despite the fact that TBW increased in males and decreased in females between the first and second serial studies, and the difference between the per cent change in TBW of males and females was significant. The validity of this interpretation of our data depends upon the validity of the proposition that there is no difference between males and females in the chemical composition of body tissue, at least with respect to potassium content. We have not been able to study clinically healthy subjects to evaluate this point. Other evidence to evaluate the proposition is meager but is available and is derived from the following sources: (1) analysis of whole adult cadavers, (2) analysis of tissue samples, (3) isotopic dilution studies, and (4) measurement of K^{40} content of human beings.

Analysis of Whole Adult Cadavers.—The reported data and their source are presented in Table V.[20-22] If we discard the 48-year-old man who was wasted and edematous and if, in addition, we assume that the fat-free body model and the lean body mass model are identical, we find nothing here to contradict our hypothesis. If the higher K_{LBM} of the female analyzed does not represent a sampling error and the K_{LBM} of females is greater than the K_{LBM} of males, then our hypothesis is further reinforced by these data, since the changes we have found are in the opposite direction.

TABLE V BODY CONTENT OF POTASSIUM OBTAINED BY DIRECT ANALYSIS OF CADAVERS

REFERENCE	SEX	AGE	K (mEq/kg) FAT FREE BASIS
Shohl Mineral Metabolism, 1939, Reinhold Publishing Corporation	Adult (Patient 19, Table III)	–	66.8
	Adult (Patient 20, Table V)	–	68
Widdowson and associates *Clin Sc* 10 113, 1951	M	25	71.1
	F	42	72.6
	M	48	33.5
Forbes and Lewis *J Clin Invest* 6 596, 1956	M	46	66.5
	M	60	66.6

Analysis of Body Tissue—A recent comprehensive review[23] of the water and electrolyte content of various body tissues does not consider possible sex differences in the chemical composition of tissues A compilation of biologic data[24] does not subclassify the data, pertaining to chemical composition of body tissues, by sex Investigators with extensive experience in the analysis of muscle[25] and nervous tissue[26] have never demonstrated any sex differences in the electrolyte content of these tissues Other workers fail to designate the sex of control subjects in their published work[27-33] and combine males and females in the statistical analysis of data When the sex is given, a significant difference in the potassium content of liver[31] and muscle[34-37] cannot be demonstrated The situation is not quite so clear cut for blood Sex differences in serum electrolyte concentration have not been demonstrated[38] While some workers have demonstrated a significantly higher red cell content of potassium in women,[39] other investigators using better sampling techniques have not been able to confirm that a difference exists[40] In summary, most workers have assumed sex differences do not exist or have not been able to demonstrate any sex difference in the potassium content of liver, brain, skeletal muscle, and blood

Isotopic Dilution Studies—Table VI summarizes isotopic dilution data published by other workers[41-46] for clinically healthy and hospital control adults

K_e/B Wt· The wide spread of the data reported for adults for each sex may be due to one or all of the following factors (1) sampling errors, (2) differences in experimental technique, or (3) true differences in body composition or potassium metabolism of individuals from different geographical regions Most authors attribute the difference between sexes to a difference in the content of body fat

K_{LBM}· The lean body mass model and the fat free body model can be considered to be identical for the purposes of our discussion for the incorporation of the concept of essential lipids into the LBM model[15] does not change the sense of our analysis Except for the data of Ikkos,[47-50] the data of different investigators are in good agreement for K_{LBM} of men The significantly lower K_{LBM} for women in these studies is surprising in view of the

Table VI. Body Content of Potassium From Isotopic Dilution Studies

REFERENCE	MALES					FEMALES				
	NO. OF SUB-JECTS	AGE IN YEARS	$\overline{K}_e$/B. WT. (mEq./KG.)	$\overline{K}_{LBM}$ (mEq./KG.)	$\overline{K}_{ICW}$ (mEq./L.)	NO. OF SUB-JECTS	AGE IN YEARS	$\overline{K}_e$/B. WT. (mEq./KG.)	$\overline{K}_{LBM}$ (mEq./KG.)	$\overline{K}_{ICW}$ (mEq./L.)
Corsa and associates: *J. Clin. Invest.* 29: 1280, 1950	29	25.8 ± 3.3	46.3 ± 4.3	–	–	–	–	–	–	–
	12	25.4 ± 3.5	–	55.9 ± 3.5	125 ± 11	–	–	–	–	–
Deane and Smith: *J. Clin. Invest.* 31: 197, 1952	5	31.4 ± 12.3	41.5 ± 3.2	56.0 ± 3.5	111.6 ± 11.1	1	18	32.9	49.8	113
Aikawa and associates: *J. Clin. Invest.* 31: 367, 1952	–	–	–	–	–	20	24.1 ± 3.2	31.5 ± 2.9	–	–
Arons and co-workers: *J. Clin. Invest.* 33: 1001, 1954	5	28.0 ± 1.4	51.1 ± 2.7	–	–	7	23.6 ± 2.4	42.5 ± 3.7	–	–
Blainey and associates: *Clin. Sc.* 13: 165, 1954	17	44.9 ± 12.0	45.9 ± 15.0	–	–	7	40.7 ± 11.1	35.0 ± 13.0		
Moore and co-workers: *Metabolism* 3: 334, 1954	–	–	–	–	–	14	23.6 (19-29)*	39.0 ± 5.4		
Ikkos and co-workers: *Metabolism* 4: 231, 1955	9	30.2 ± 7.1	50.6 ± 9.3	67.5 ± 4.3	127.1 ± 6.9†	5	28.4 ± 7.7	38.7 ± 4.3	58.2 ± 6.7	113.7 ± 14.4†

Rundo and Sagild *Nature* 175 774, 1955	6	28 2± 4 2	48 6± 2 2	–	–	4	21 2± 5 0	42 7± 2 9		
Corsa and associates *Pediatrics* 17 184, 1956	1	0 083 (prema ture)	40 0	–	–	–	–	–	–	–
	8	0 82±0 82	37 5± 3 7	38 6±3 0‡	–	–	–	–	–	–
	2	11 0± 5 9	40 0± 1 4	45 3±1 3	–	5	10 2± 3 0	38 5± 9 4	46 6±3 6§	–
Ikkos and co workers ‖ *Acta Endocrinol* 21 211, 1956	13	30 4± 8 5	50 2± 6 2	61 6±3 3	116 9±6 9**	19	25 8± 6 9	37 9± 4 2	55 4±4 2	107 6± 8 7**
James and Robertson *A M A Am J Dis Child* 93 217, 1957 (Sex not stated)	7	5 9± 2 2	44 1± 2 9	57 2±6 5	–	–	–	–	–	–
Muldowney and associates *J Clin Invest* 36 1375, 1957	13	29 4±10 1	45 7± 6 2	57 2±4 7	–	10	48 2±17 8	30 1± 3 6	46 3±7 2	–

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*Individual ages not listed

†Using TBW$_{AP}$ and ECW$_{RN}$ for Subjects 1 to 12 TBW$_{AP}$ and ECW$_{TS}$ for Subjects 13 and 14

‡Seven subjects

§Four subjects

‖Probably includes subjects reported in 1955

**Using ICW$_{TS}$

fact that in our studies K_{LBM} was experimentally identical in males and females by the third serial study. In the study of Muldowney and his co-workers[52] this difference could be due to the difference in age of the male and female subjects, for age variations in muscle electrolyte composition have been described.[53] Corsa and co-workers[41] have stated K^{42} exchanges with 95 per cent of the body potassium. A limited number of measurements of K^{42} and K^{40} in the same subject by Rundo and Sagild[48] has indicated $\frac{K^{42}}{K^{40}} = 85$ per cent. If we apply this ratio to the available data for K^{42}_{LBM} for males we find, with the exception of the results of Ikkos,[47] the corrected K^{42}_{LBM} data to be in fairly good agreement with the results obtained from the direct analysis of cadavers. For females the data of Ikkos are of the right order of magnitude while Muldowney's data are low. As mentioned previously we cannot explain these discrepancies at the present time.

Experimental support for the extrapolation of the regression line toward the origin is available in Table VI. The data for infants indicate a K_{LBM} somewhat lower than that found for adults. However, this is probably due to the appreciably larger extracellular fluid volume found in infants.[54] Correction of the $\overline{K}_{LBM}$ data for infants for this enlarged ECF brings their values into the adult range.

K_{ICW}: The variation in K_{ICW} for each sex can be accounted for in part by the use of substances, for the measurement of body fluid compartments, with noncongruent volumes of distribution. However, the sex difference in Ikkos' data is significant ($P < 0.05$) and is as surprising as his K_{LBM} data, for there was no significant ($P > 0.05$) difference in K_{ICW} between our male and female subjects by the third serial study.

K^{40} Content of Humans.—A number of workers[48, 55-61] have been interested in the K^{40} content of humans. Burch and Spiers[60] have summarized much of the data: for males, 30 mEq./Kg. $< K^{40}/\text{B. Wt.} <$ 55 mEq./Kg.; for females, 30 mEq./Kg. $< K^{40}/\text{B. Wt.} <$ 51 mEq./Kg. On the basis of fat-free weight the potassium content of the body appears to be independent of gross weight, age, and sex and is estimated to be 71.6 mEq./Kg. of fat-free weight.[58, 61] This value is probably the most reliable estimate of the fat-free body content of potassium, for the studies have been limited to clinically healthy individuals. In addition, it would indicate a ratio for $\frac{K^{42}}{K^{39}}$ in the neighborhood of the 85 per cent indicated by Rundo and Sagild,[48] and somewhat lower than the 95 per cent suggested by Corsa and associates.[41]

In conclusion, we would interpret the available evidence as indicating an identical body content and tissue concentration of potassium in clinically healthy males and females. One may question the validity of an estimation of the composition of tissue of clinically healthy subjects obtained by extrapolation from the data of our third serial study. However, in an experimental model similar in many respects to our clinical situation, Eichelberger[62] has shown that in muscle the intracellular concentration of potassium does not change and the extracellular fluid volume increases after denervation Unfortunately she did not study the dynamics of the change

Our observation of an increase in K_{LBM} and K_{ICW} in males can be used to explain the interesting results of Howard[63, 64] and Whedon and Shorr[65] of potassium losses considerably less than the theoretical losses expected during balance studies Our failure to observe any significant correlation between $[K]_s$ and K^{42}, K_{LBM}, or K_{ICW} confirms the observations of others[66], however we did not measure serum pH or pCO_2 as have others[67, 68] Our failure to do so may have precluded the observation of any relationship between $[K]_s$ and pH, pCO_2, K_e^{42}, K_{LBM}, or K_{ICW} Other studies of ours indicate a relationship between LBM and $[K]_s$ and because of the linear relationship between K_e and LBM suggest a relationship between K_{LBM} and $[K]_s$[69]

The water metabolism of the patients studied serially is of interest Rigid fluid and sodium restriction was enforced for these patients despite the fact that they were acutely ill with high body temperatures, had continuous nasopharyngeal suction, and were encased in tank respirators—conditions which presumably contributed to the induction of maximal fluid losses Clinical studies[12] of patients during previous epidemics suggest that the presence of pulmonary edema at post mortem was associated with the conventional regimen of 3 L of water and 8 5 Gm of NaCl administered daily The demonstration of an actual increase in the TBW in the males and the small decrease in TBW in the females between the first and second studies despite the rigid fluid restriction provides experimental support for the previous clinical conclusions Unfortunately, the physical conditions of the epidemic precluded clear cut documentation of this point in terms of fluid balance However, the large quantities of water that can be produced by catabolism of body tissues has been documented in other conditions[14, 70] The changes in water distribution are identical to those that have been described in undernutrition[71, 72] This suggests that these changes in water distribution may be one facet of a general biologic response to stress The use of the LBM model assumes that the ratio $\frac{\text{cell mass}}{\text{total body water}}$ remains constant It is evident that this was not so in our studies The application of any correction factor to take this into account would accent the differences in K_{LBM} and K_{ICW}, in view of the fact that initially the TBW for males increased while the TBW for females decreased

There remains for discussion the biologic interpretation of our observations Analysis of the epidemiologic and clinical data indicates that young men are less susceptible to infection with poliomyelitis virus, have a higher incidence of complications and have a higher mortality than nonpregnant young women[73, 78] The simplest assumption to account for these differences is to relate them to the different steroid hormones present in the internal environment of males and females Support for this proposition as a first approximation is obtained from the work of Teodoru and Shwartzman, who have been able to modify the susceptibility to infection with poliomyelitis virus of the Golden Syrian hamster (normally a resistant animal) by suitable alteration of testicular and adrenocortical function.[79-82] Certain of their experiments suggest that simultaneous administration of testosterone and cortisone to hamsters increases the paralytic incidence and mortality following intracerebral inoculation of MEF virus, when compared to control, cortisone-treated, testosterone-treated, and castrated animals.[79, 83] The important role that changes in the physicochemical environment of the tissues of the host play in the modification of the host-parasite relationship has been recently reviewed and discussed by Dubos.[84] The importance of hormonal factors in this scheme is well illustrated by the enhanced susceptibility of women to infection with poliomyelitis virus produced by pregnancy.[74, 76]

Studies are in progress to delineate further the changes in the internal environment that occur during the course of acute central nervous system infections.

SUMMARY

1. Total exchangeable potassium, antipyrine space, and radiosulfate space were simultaneously determined in 44 patients suffering from acute anterior poliomyelitis. In a selected number of patients the measurements were repeated at 7- to 10-day intervals.

2. A significant difference in potassium metabolism between males and females was demonstrated. In males the intracellular potassium concentration was increased early in the illness, while in females it remained unchanged during the course of the illness.

3. A significant difference in the metabolism of water between males and females was demonstrated. In males total body water increased early in the illness while in females it decreased during the course of the illness. The distribution of water between intracellular and extracellular compartments was identical in males and females. During the course of the illness the extracellular fluid compartment expanded and the intracellular fluid compartment contracted.

4. The biologic interpretation of these sex differences in potassium and water metabolism is discussed.

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THE EFFECT OF DIGITALIS AND DESOXYCORTICOSTERONE ACETATE ADMINISTRATION UPON INTRACELLULAR POTASSIUM ION CONCENTRATION

A Chemical and Morphologic Study

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THE theory of Szent-Gyorgi[1] on muscle contraction has drawn attention to the importance of potassium in intracellular physiology. Since the publication of his work, it has become increasingly evident that the actions of digitalis are intimately associated with the intracellular concentration of the potassium ion.[2]

It has been well documented that toxic doses of digitalis are capable of producing myocardial lesions in experimental animals.[3-5] Numerous investigators have indicated also that potassium deficiency results in similar morphologic changes both in animals and in man.[6-8] As a corollary to these studies it has been shown that potassium depletion in animals increases cardiac sensitivity to digitalis.[9-11] Robinson and associates[12] have recently stated that in potassium-deficient rats toxic doses of digitalis did not accentuate the development of myocardial lesions. These authors felt that digitalis neither added to nor protected from the effect of potassium deficiency in their animals.

In this study large doses of digitoxin alone and in combination with desoxycorticosterone acetate (DOCA) were administered to dogs. The results of a chemical and morphologic study of the myocardium of these animals form the basis for the present report.

METHODS

Mongrel dogs were used throughout the study. A standard laboratory diet was given, and water was allowed as desired. All drugs were administered by intramuscular injection daily except on Saturday and Sunday. The animals receiving digitoxin lost weight, became anorexic, and appeared toxic at the end of the experimental period.

The animals were divided into 4 groups.

Group I. Controls: Four dogs considered to be in good health.

Group II. DOCA Alone: Four dogs, 2 of which received 15 mg. of DOCA daily for a total dose of 150 mg. in 13 days and 2 dogs received a total dose of 225 mg. in 20 days.

Group III. Digitoxin Alone: Two dogs received 0.4 mg. daily for a total dose of 6 mg. in 20 days and 3 animals received 0.6 mg. daily for a total dose of 9 mg. in 20 days.

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Group IV DOCA Plus Digitoxin Five animals received 0.6 mg digitoxin daily plus 15 mg of DOCA daily for a total dose of 9 mg of digitoxin and 225 mg of DOCA in 18 to 21 days, respectively

All animals were killed by ether anesthesia Blood was removed immediately from the left ventricle by means of syringe and needle for estimations of serum sodium potassium and chloride The hearts were then removed for the following pathologic and chemical studies

Tissue blocks were taken from the anterior, lateral, and posterior ventricular walls right ventricle, and interventricular septum A minimum of 6 tissue blocks were selected from each heart Tissues were fixed in 10 per cent formalin Microscopic sections were cut at 6 micra and stained with hematoxylin and eosin and by the Hotchkiss McManus *periodic acid Schiff stain*

Chemical analysis was performed on portions of the left ventricular myocardium in each case Tissue specimens varied in weight between 0.5 to 1.0 Gm One tissue specimen was dried in an oven at 110° C to constant weight to determine dry weight The dried tissue was then extracted with ether to determine fat content, fat free wet muscle weight and fat free dry muscle weight In each case 5 additional muscle specimens were taken for chemical analysis The values for each dog represent the mean of these 5 analyses After weighing, each muscle specimen was homogenized in 50 ml of distilled water using a Waring blender with microcell After homogenization, the mixture was centrifuged at 3 000 r p m for 10 minutes to produce a slightly opalescent flesh colored fluid Complete disintegration of muscle fibers was determined by microscopic examination of the homogenate Chlorides were determined on a 20 ml aliquot using the method of Whitehorn[13] Sodium was determined on undiluted samples and potassium on a 1 25 dilution of the sample by means of flame photometry A Beckman Model B spectrophotometer with flame attachments was used exclusively

Extracellular water was determined from tissue chlorides and serum chloride according to standard methods and formulas[14] The following symbols are used in the tables

H_2O_m = grams of muscle water per kilogram of wet fat free muscle
Cl_m = mEq of chloride per kilogram of wet fat free muscle
H_2O_e = extracellular phase in gram of water per kilogram of wet fat free muscle
H_2O_i = intracellular phase in grams of water per kilogram of wet fat free muscle
Na_i = mEq of Na per kilogram of wet fat free muscle
K_i = mEq of K per kilogram of wet fat free muscle
Na_{cw} = mEq of Na per kilogram of intracellular water
K_{cw} = mEq of K per kilogram of intracellular water
Na_s = serum Na in mEq per liter of serum
K_s = serum K in mEq per liter of serum
Cl_s = serum Cl in mEq per liter of serum

RESULTS

A *Tissue Analysis Data*—The noteworthy findings of Table I are the following

(1) In Groups II (DOCA alone), III (digitoxin alone), and IV (DOCA plus digitoxin), H_2O_e is increased and H_2O_i is decreased In these same groups K_i is decreased and K_s is increased (2) In Groups III (digitoxin alone) and IV (DOCA plus digitoxin), K_{cw} is increased despite the decrease in K_i

These data indicate that both DOCA and digitoxin produced a decrease in intracellular water and an increase in extracellular water in the myocardium It is interesting to note that during the course of this study the serum potassium concentration rose It should be emphasized that due to the decrease of intracellular water the actual ionic concentration of intracellular potassium, K_{cw}, increased in the animals receiving digitoxin alone as well as in those receiving digitoxin plus DOCA.

TABLE I. SUMMARY OF THE MEAN RESULTS OF THE CHEMICAL STUDIES FOR ALL FOUR GROUPS

	GROUP I CONTROL		*GROUP II* DOCA ALONE		*GROUP III* DIGITOXIN ALONE		GROUP IV DOCA PLUS DIGITOXIN	
NO. DOG	4	VARIATION	4	VARIATION	5	VARIATION	5	VARIATION
H_2O_m	749	740-755	724	705-735	743	722-774	747	730-761
Cl_m	19.5	18.2-20.3	21.7	19.7-23.8	25.9	24.6-27.3	32.3	25.5-37.3
H_2O_e	152	146-157	168	153-179	199	184-209	240	188-278
H_2O_i	597	583-609	556	552-558	554	527-570	507	463-548
Na_i	7.6	4.7-11.1	10.3	9.0-12.5	8.1	6.4-12.3	9.0	4.9-10.7
K_i	81.3	76.1-83.6	75.3	72.5-78.1	78.0	72.1-82.5	71.7	61.5-79.5
Na_s	148	146-151	154	150-158	156	148-164	158	151-164
K_s	5.6	4.8-6.0	7.6	5.8-10.0	7.4	5.9-8.9	7.2	5.8-8.7
Cl_s	112	109-113	113	110-116	114	112-117	117	112-119
Na_{cw}	12.6	8.1-18.1	18.5	16.0-23.0	14.9	10.0-23.0	17.7	9.8-20.3
K_{cw}	136.6	125-143	136.0	130-143	143.5	134-153	141.0	125-169

B. *Morphologic Data.*—Microscopic examination of tissue from all animals revealed no characteristic changes. Several sections from each animal showed patchy areas of lymphocytic infiltrations, interstitial fibrosis and some minimal areas of cellular necrosis. These findings appeared occasionally in the control animals as in the other groups. The administration of DOCA and excessive doses of digitoxin and the chemical aberrations produced by these substances did not cause any significant degree of abnormal morphologic change.

DISCUSSION

From the results of this study it is apparent that DOCA alone and the combination of DOCA and toxic doses of digitoxin are capable of lowering intracellular potassium content. Digitoxin alone did cause a fall in intracellular potassium, but not to a marked degree. In contrast to our findings, Gardner[15] and his associates found in the rat that the combination of DOCA and strophanthin G did not lower intracellular potassium to as low a level as when either agent was used alone. These authors concluded that a metabolic antagonism exists between digitalis and potassium. This may be explained by the observation of Robinson[12] that the rat is resistant to the effects of digitalis. Zeeman and associates,[10] on the other hand, found that potassium-depleted dogs exhibited a marked sensitivity to lanatoside C, but were unable to demonstrate a definite decrease in cardiac muscle potassium content.

Most important in the present study is the fact that although intracellular potassium levels diminished, intracellular water content also diminished. This resulted in an actual increase in potassium ion concentration per unit of intracellular water in the dogs that received digitoxin alone and those that received digitoxin plus DOCA. Lown[2] has stated, "There is general agreement that toxic doses of digitalis liberate potassium from both skeletal and heart muscle." In our study, excessive doses of digitoxin did produce some decrease in total cardiac muscle potassium, but of greater significance was the actual increase in intracellular potassium ion concentration.

If it is ever permissible to correlate experimental data with clinical experience, the results of this study would imply that the effectiveness of potassium administration to patients suffering from digitalis intoxication may not be dependent upon the increase in the intracellular concentration of the potassium ion

The lack of clear cut morphologic changes in the myocardium following DOCA and digitoxin administration may be explained by the rather short duration of the experiment, and by the absence of protein depletion or the lack of an excessive sodium intake These factors may be necessary concomitants for the production of potassium deficiency lesions in the dog

SUMMARY

A study is presented in which groups of dogs were given injections of large doses of DOCA and digitoxin Chemical analysis of the heart muscle of these animals revealed that DOCA alone, digitoxin alone, and a combination of these agents produced a drop in total muscle potassium However, due to the decrease in intracellular water content, actual intracellular potassium ion concentration was increased in the animals receiving digitoxin alone and those receiving digitoxin plus DOCA

The animal hearts were studied for morphologic lesions similar to those reported elsewhere, but no difference was found in the drug treated animals from a group of normal controls

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THE ABSORPTION OF VITAMIN B_{12} LABELED WITH RADIOACTIVE COBALT^{60} FOLLOWING SUBTOTAL GASTRECTOMY

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MEGALOBLASTIC anemia is an uncommon complication of a subtotal gastrectomy,[1, 2] although there have been reports of such an occurrence.[3-9] Recent reports indicate that impaired absorption of vitamin B_{12} occurs following subtotal gastrectomy, but is uncommon.[5, 9-12] We have performed a similar study of the absorption of Co^{60}-labeled vitamin B_{12} in a large group of patients who had had subtotal gastrectomies. In patients with impaired absorption of vitamin B_{12}, the effect of gastric stimulation with a secretagogue and the effect of the administration of an intrinsic factor preparation have been studied. In addition, we have investigated the role of the type of anastomosis involved in the production of the absorptive defect.

METHODS AND MATERIALS

The absorption of Co^{60}-labeled vitamin B_{12} was measured by means of a modification of the Schilling test,[13] the results being expressed as the per cent of the radioactivity of the oral dose (1 μg vitamin B_{12} labeled with 0.7 to 1.0 μc Co^{60}) excreted in the urine in 24 hours. In 16 normal subjects and in 17 patients with pernicious anemia the range of values for the 24-hour urinary excretion of radioactivity was found to be 6.5 to 22.3 per cent and 0.0 to 1.3 per cent, respectively. Ten patients with pernicious anemia had the Schilling test repeated with administration of intrinsic factor concentrate at the time of the oral dose of Co^{60}-labeled vitamin B_{12} and the range of values was found to be 4.0 to 19.7 per cent.

The absorption of vitamin B_{12} was measured in 34 patients who had undergone conventional subtotal gastrectomy from 1 to 70 months earlier. Fifteen patients had subtotal gastric resections with gastroduodenostomies, and 19 patients had subtotal gastric resections with gastrojejunostomies. Vagotomies had been performed in 2 patients in the former group and 4 patients in the latter. In both groups of patients, 60 to 80 per cent of the distal portion of the stomach had been resected, leaving a portion of the fundus in every case. Surgery was performed for peptic ulcer in 30 instances and for carcinoma in 4. Anemia was not present in any of the patients.

Four patients with subtotal gastrectomies were found to have an abnormally low urinary excretion of Co^{60}-labeled vitamin B_{12} comparable to values found in cases of pernicious anemia. Additional studies were performed on these patients as follows:

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The intrinsic factor concentrate used in these studies was supplied through the courtesy of Dr. Kenneth W. Thompson of Organon, Inc., Orange, N. J.

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(1) a Schilling test with concomitant administration of 50 mg of intrinsic factor concentrate, (2) a Schilling test 90 minutes after gastric stimulation with 50 mg of Histalog (3 beta aminoethyl pyrazole) administered subcutaneously[14], (3) blood urea nitrogen and blood creatinine determinations, (4) plasma prothrombin and serum carotene levels. The latter procedures were performed to eliminate renal disease and small bowel malabsorption syndromes.

RESULTS

The results are presented in Tables I and II. The range of urinary excretion of Co^{60}-labeled vitamin B_{12} was 7.5 to 36.4 per cent in the group of 15 patients with subtotal gastric resection with gastroduodenostomy. Three patients had associated vagotomies.

The range of urinary excretion of Co^{60}-labeled vitamin B_{12} was 0.7 to 31.3 per cent in the group of 19 patients with subtotal gastric resection and gastrojejunostomy. Four of the patients in this group excreted less than 4.7 per cent of the administered radioactivity. In all 4 patients the urinary excretion of radioactivity increased to within the normal range when the Schilling test was repeated with 50 mg of intrinsic factor concentrate. In 2 of these 4 patients urinary excretion of vitamin $B_{12}Co^{60}$ increased after Histalog administration, one rising to 6.4 per cent. Blood urea nitrogen, blood creatinine, plasma prothrombin concentration, and serum carotene determinations were normal in these patients.

DISCUSSION

Impaired absorption of vitamin B_{12}, as measured by the Schilling test of urinary excretion, was found in 11 per cent of a group of 34 patients who had had subtotal gastric resections. The patients who had impaired absorption of vitamin B_{12} were not anemic probably because they were studied too soon after gastrectomy to have depleted their body stores of vitamin B_{12}. Megaloblastic anemia is uncommon following subtotal gastric resection. MacLean in screening over 1,500 patients with subtotal gastrectomies found only 9 patients with megaloblastic anemia.[9]

The 4 patients with impaired absorption of vitamin B_{12} had had subtotal gastric resections with gastrojejunostomies. None of the 15 patients, who had subtotal gastric resections with gastroduodenostomies had impaired absorption of vitamin B_{12}. Twenty five cases of megaloblastic anemia following subtotal gastric resection or gastrojejunostomy have been reported in the medical literature *with details of the type of surgical anastomosis*[3, 9] *In all but one of* these cases, the subtotal gastric resection has been associated with a gastrojejunostomy. In the one case associated with gastroduodenostomy, a high Billroth I resection was accomplished.[5] The 3 patients reported by Lowenstein[12] whose absorption of vitamin B_{12} was impaired after subtotal gastric resection also had had gastrojejunostomies.

These observations indicate that impaired absorption of vitamin B_{12} after subtotal gastric resection occurs predominately in patients who have had the stomach remnant anastomosed to the jejunum. Since the defect in absorption

TABLE I

PATIENT	AGE	DIAGNOSIS	MONTHS AFTER OPERATION	TYPE OF PROCEDURE	GASTRIC ANALYSIS BEFORE OPERATION	PER CENT URINARY EXCRETION OF VITAMIN $B_{12}Co^{60}$
						VITAMIN $B_{12}Co^{60}$ ALONE
J. J.	54	Gastric ulcer	19	Gastroduodenostomy	Free acid with histamine	11.0
E. N.	77	Duodenal and gastric ulcer	43	Gastroduodenostomy	Free acid	9.6
F. M.	50	Duodenal ulcer	39	Gastroduodenostomy and vagotomy	Free acid	21.5
C. W.	57	Carcinoma of stomach	1¼	Gastroduodenostomy	—	8.5
E. S.	65	Duodenal ulcer	21	Gastroduodenostomy	Free acid postoperatively	30.1
A. P.	60	Duodenal ulcer	30	Gastroduodenostomy	—	15.8
M. W.	50	Duodenal ulcer	6	Gastroduodenostomy	Free acid after histamine	11.7
B. H.	39	Duodenal ulcer	7	Gastroduodenostomy	—	10.4
M. H.	58	Carcinoma of stomach	19	Gastroduodenostomy	—	9.6
D. A.	56	Gastric ulcer	13	Gastroduodenostomy	—	13.0
W. B.	62	Gastric ulcer	2	Gastroduodenostomy	Free acid with histamine	7.5
P. D.	51	Gastric ulcer	20	Gastroduodenostomy	Free acid	20.0
W. M.	18	Gastritis, no ulcer	3	Gastroduodenostomy and vagotomy	Free acid	18.8
G. M.	35	Duodenal ulcer	16	Gastroduodenostomy	Free acid	24.6
J. R.	34	Duodenal ulcer	1¼	Gastroduodenostomy	—	36.4
C. P.	39	Pyloric ulcer	1	Gastrojejunostomy	Free acid	25.1
N. W.	42	Chronic gastritis	7	Gastrojejunostomy	—	9.7
B. A.	65	Duodenal ulcer	4	Gastrojejunostomy and vagotomy	Free acid	22.9
F. G.	82	Duodenal ulcer	2¾	Gastrojejunostomy	—	12.4
M. S.	56	Gastric and duodenal ulcer	6¼	Gastrojejunostomy	—	18.0
C. W.	48	Duodenal ulcer	4	Gastrojejunostomy	No free acid after histamine	11.7
C. C.	70	Gastric and duodenal ulcer	3½	Gastrojejunostomy	—	25.6
C. T.	65	Gastric ulcer	15¾	Gastrojejunostomy	Free acid after histamine	17.8
J. M.	58	Duodenal ulcer	28¼	Gastrojejunostomy	—	15.8
A. D.	47	Duodenal ulcer	5¾	Gastrojejunostomy	—	17.1
F. F.	61	Duodenal ulcer	4¾	Gastrojejunostomy	—	23.2
S. F.	49	Carcinoma of stomach	5	Gastrojejunostomy	—	31.3
J. B.	40	Duodenal ulcer	60	Gastrojejunostomy	—	10.5
E. G.	30	Duodenal ulcer	70	Gastrojejunostomy	—	8.2
G. D.	71	Duodenal ulcer	9	Gastrojejunostomy	—	18.5

of vitamin B_{12} can be corrected by the addition of intrinsic factor the abnormality can be attributed to an inadequate quantity of intrinsic factor Several factors undoubtedly contribute to this inadequacy First decreased gastric secretion of intrinsic factor may occur, because of an inadequate gastric phase of secretion Stimulation of gastric secretion through the gastric mechanism can be invoked by mechanical distention of the pyloric antrum by food or by contact with the antral mucosa of chemical substances derived chiefly from meat The resection of a large segment of the antrum associated with increased rate of gastric motility and emptying may decrease the effectiveness of this hormonal mechanism In 2 of our cases with poor absorption of vitamin B_{12} stimulation of gastric secretion with a secretagogue resulted in improved absorption of vitamin B_{12}

TABLE II

PATIENT	AGE	DIAGNOSIS	TIME IN MONTHS AFTER OPERATION	TYPE OF PROCEDURE	GASTRIC ANALYSIS BEFORE OPERATION	PER CENT URINARY EXCRETION OF VITAMIN B_{12} Co^{60} AFTER: VITAMIN B_{12} Co^{60}	PLUS INTRINSIC FACTOR	FOLLOWING 50 MG HISTALOG
C R	33	Duodenal ulcer, diverticulum of third portion of duodenum	1½	Gastrojejunostomy and vagotomy	Free acid	3 8	7 2	6 4
M H	60	Carcinoma of stomach	7	Gastrojejunostomy	—	0 7	7 9	0 7
T S	64	Duodenal ulcer	½ mo with intrinsic factor 1 mo with Histalog	Gastrojejunostomy and vagotomy	—	4 7	10 4	4 6
L R	44	Duodenal ulcer	1¼	Gastrojejunostomy	—	1 8	12 4	5 6

In the 9 cases of megaloblastic anemia associated with subtotal gastric resection reported by MacLean,[9] he noted in the surgical specimens intestinalization of the gastric mucosa and considered this a cause for decreased secretion of intrinsic factor Three of the 4 patients with decreased absorption of vitamin B_{12} who were studied in our laboratory had normal gastric mucosa in the surgical specimens The fourth patient (M H) was found to have a gastric carcinoma with intestinalization of the gastric mucosa

Second, following gastric resection with gastrojejunostomy the duodenum is bypassed, and the stomach contents enter directly into the jejunum Although vitamin B_{12} in the presence of intrinsic factor is absorbed throughout the small intestine, the duodenum is undoubtedly one of the more important sites of vitamin B_{12} absorption Citrin[15] and his associates, in studies on the site of absorption of vitamin B_{12} from the intestinal tract in normal subjects obtained the highest urinary excretion values when vitamin B_{12} was instilled by tube directly into the duodenum although significant absorption of vitamin B_{12} did occur following instillation into the jejunum and ileum. Observations of von Bonsdorff[16] in cases of megaloblastic anemia associated with fish tapeworm infestation also attest to the importance of the duodenum in vitamin B_{12} absorption. Dislodgment of the worms from the duodenum induced remission of the megaloblastic anemia even though the tapeworms remained in the lower portion of the intestinal tract.

Naish[17] and his associates reported a case of megaloblastic anemia following gastrojejunostomy and attributed the cause to intestinal stagnation in the afferent loop of jejunum. In our cases the blind loop syndrome is an unlikely explanation for the defect in absorption of vitamin B_{12} inasmuch as the defect was corrected by the simultaneous administration of intrinsic factor and vitamin B_{12}. In the presence of intestinal blind loops which interfere with vitamin B_{12} absorption, intrinsic factor does not correct the absorptive defect.

Intestinal malabsorption of vitamin B_{12} associated with steatorrhea also is unlikely in our patients inasmuch as intrinsic factor corrected the absorptive defect. In addition, the 4 patients had no diarrhea and maintained their weights. Plasma prothrombin and serum carotene values were normal in all cases.

Renal disease as a cause of an abnormally low urinary excretion of vitamin B_{12} was excluded since there was no history of renal disease, the urinalyses, blood urea nitrogen, and creatinine values were normal and the low urinary excretion of Co^{60}-labeled vitamin B_{12} returned to normal levels with the addition of intrinsic factor to the Schilling test.

SUMMARY

Vitamin B_{12} absorption was studied by means of a modified Schilling technique in 34 patients who had had subtotal gastric resection. Decreased absorption was found in 4 of the 19 patients who had had subtotal gastric resections with gastrojejunostomies. The deficiency was corrected by the administration of intrinsic factor. All of the patients with gastroduodenostomies absorbed vitamin B_{12} normally. Decreased gastric secretion of intrinsic factor and bypassing of the duodenum are considered important factors in the decreased absorption of vitamin B_{12} in the patients with subtotal gastrectomy associated with a gastrojejunostomy.

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D-XYLOSE AND POTASSIUM IODIDE ABSORPTION AND SERUM CAROTENE IN PERNICIOUS ANEMIA

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THE presence of an absorptive defect in pernicious anemia was initially demonstrated by Castle[1] who, in 1929, described the absence of intrinsic factor in the gastric juice. Citrin and associates,[2] instilling vitamin B_{12} directly into the small intestine, have shown this to be the normal site of B_{12} absorption. Employing a modified Schilling test, they further confirmed the inability of patients with pernicious anemia to absorb vitamin B_{12} in the absence of added intrinsic factor.

The question of impaired small intestinal absorption of other nutrients in pernicious anemia is raised by certain similarities of this disease to nontropical sprue. Megaloblastic anemia, glossitis, peripheral neuropathy, and low serum vitamin B_{12} levels are frequently encountered in sprue patients.[3-5] In addition, Gardner[6] has described similar alterations in gastric cytology in these two conditions.

Depression of serum carotene levels[7] and xylose absorption[8, 9] are found in the sprue syndrome. Heath and Fullerton[10] noted a delay in salivary iodide excretion following oral ingestion of potassium iodide in 7 of 14 patients with pernicious anemia, and concluded that this was evidence of impaired iodide absorption from the small intestine. It is the purpose of this report to evaluate further the absorptive activity of the small intestine in pernicious anemia.

MATERIALS AND METHODS

Serum carotene levels, blood xylose levels, and urinary excretion of xylose were measured in the following subjects:

1. Fifteen normal subjects between the ages of 25 and 72 years (Table I).
2. Twenty patients with pernicious anemia in hematologic remission, aged 55 to 85 years. The potassium iodide test was performed on 12 of this group (Table II).
3. Five pernicious anemia patients in hematologic relapse, the diagnosis in each case being confirmed by the characteristic bone marrow, low serum vitamin B_{12} levels, and a reticulocyte response to parenteral vitamin B_{12} therapy. Repeat studies were done following therapeutic remission (Table III). The potassium iodide test was also performed on 3 of these patients.

From the Gastrointestir [illegible] -tory, Mallory Institute of Pathology, and the 2nd and 4th (Harvard) [illegible] ston City Hospital, and the Department of Pathology, Boston University [illegible] Boston Mass.

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4. Six patients with chronic nonmegaloblastic anemia, with hemoglobin levels comparable to those found in Group III (Table IV).

D-Xylose and Carotene Tests.—Following an overnight fast, the bladder was emptied and the urine discarded. Twenty-five grams of D-xylose, dissolved in 500 ml. of water, was given by mouth at 7 A.M. The xylose content of a 5 hour urine collection was determined. Patients remained fasting during the test. Blood was drawn for xylose and carotene determinations 2 hours following ingestion of the sugar. Xylose was determined by the method of Roe and Rice[11], carotene by the method of Bessey and associates.[1]

Potassium Iodide Test.—The subjects drank a solution of 0.25 Gm. of potassium iodide in 50 ml. of water after an overnight fast. The mouth was then rinsed and saliva collected every 2 minutes in a cup containing 1 ml. of saturated starch solution and 4 drops of 10 per cent ferric chloride solution. The time elapsing between ingestion of potassium iodide and its appearance in saliva was measured. The presence of as little as 0.027 mg. of free iodine released by ferric chloride resulted in the appearance of a blue-black precipitate.

RESULTS

The five hour urinary xylose excretion of 10 normal subjects ranged from 3.7 to 7.9 Gm. with an average value of 5.71 Gm. (Table I and Fig. 1). The 2 hour blood levels varied from 18.2 to 42.2 mg. per cent with an average of 31.8 mg. per cent (Table I and Fig. 2). These latter values are in accord with those reported by previous authors.[9, 11, 14] Serum carotene in the control group ranged from 55 to 192 μg per cent with an average of 124.8 μg per cent (Table I and Fig. 3), which is in agreement with normal values (50 to 420 μg per cent) reported by others.[7] Salivary iodide excretion time, after oral potassium iodide ingestion, in 45 healthy subjects varied from 11 to 16 minutes.

TABLE I. SERUM CAROTENE AND D-XYLOSE DETERMINATIONS IN CONTROL SUBJECTS

CASE	AGE	2 HOUR BLOOD XYLOSE (MG. PER CENT)	5 HOUR URINE XYLOSE (GM.)	SERUM CAROTENE (μG PER CENT)
1	25	34.4	6.7	192
2	28	–	–	167
3	28	–	–	178
4	29	42.2	3.7	
5	30	36.4	7.6	108
6	30	22.6	7.9	–
7	34	–	–	123
8	36	21.5	6.8	
9	40	18.2	3.8	–
10	40	39.7	3.8	–
11	45	–	–	55
12	50	33.8	5.6	–
13	58		–	65
14	60	37.8	7.5	116
15	72	32.2	3.7	119

Normal 2 hour blood xylose levels were obtained in all 20 pernicious anemia patients in remission (Fig. 2). Urinary xylose excretion was normal in 13 of this group; low values were noted in the remaining 7 (Cases 1 to 7, Table II). Serum carotene and salivary iodide excretion times were normal in all patients tested (Table II).

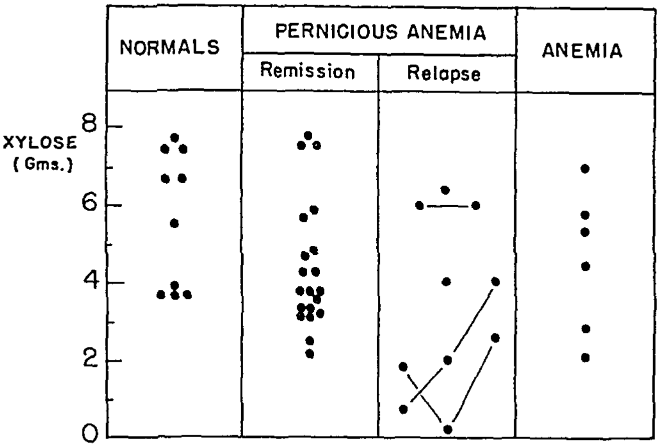

Fig. 1.—Five-hour urine xylose determinations in normal subjects and patients with pernicious and other anemias.

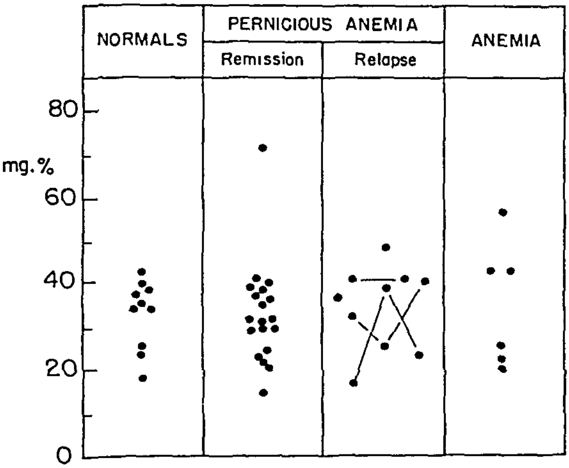

Fig. 2.—Two-hour blood xylose determinations in normal subjects and patients with pernicious and other anemias.

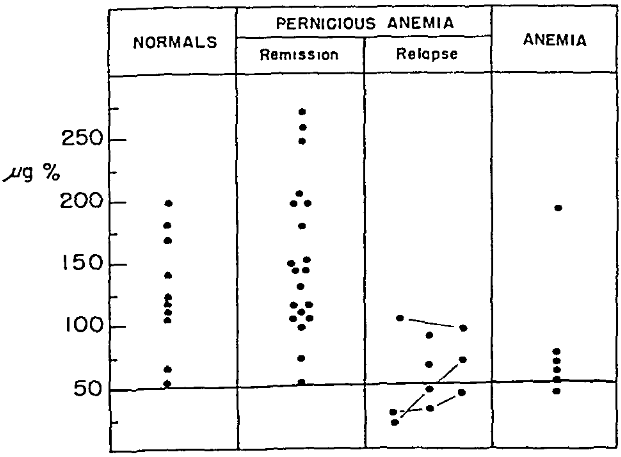

Fig. 3.—Serum carotene determinations in normal subjects and patients with pernicious and other anemias.

TABLE II. SERUM CAROTENE AND D-XYLOSE DETERMINATIONS IN PERNICIOUS ANEMIA PATIENTS IN REMISSION

CASE	AGE	HEMOGLOBIN	POTASSIUM IODIDE SALIVARY EXCRETION TIME (MINUTES)	2 HOUR BLOOD XYLOSE (MG. PER CENT)	5 HOUR URINE XYLOSE (GM.)	SERUM CAROTENE (μG PER CENT)
1 D C	72	14.0	12	*	2.1	115
2 H F	69	13.4	10	37.5	2.4	55
3 H W	77	12.5	15	23.8	3.4	99
4 C S	53	15.6	18	44.9	3.4	71
5 M O'T	54	14.1	12	40.7	3.3	265
6 G M	60	13.9	11	29.0	3.3	193
7 E S	71	12.5	10	29.9	3.2	139
8 G S	72	13.1	14	31.1	4.9	150
9 M D	55	13.2	12	35.5	5.9	115
10 R B	50	13.3	16	72.2	7.4	206
11 C B	65	14.3	14	40.0	4.7	131
12 J C	60	15.3	10	19.2	8.4	199
13 E D	71	14.2	–	21.5	5.8	138
14 J G	65	16.8	–	38.1	4.2	249
15 I H	50	14.5	–	32.1	3.8	148
16 M L	65	15.6	–	20.6	7.5	171
17 R S	60	13.2	–	35.7	3.8	116
18 G E	75	14.5	–	31.6	4.2	112
19 E DeP	71	14.3	–	29.1	3.8	108
20 J T	62	13.5	–	37.8	3.7	254

*Determination not made because of elevated (49 mg. per cent) NPN.

Three of 5 pernicious anemia patients in relapse had normal values for blood and urine xylose and serum carotene. Diminished urinary excretion of xylose was noted in the remaining 2 (Cases 1 and 2, Table III), but increased as the hematocrit rose on vitamin B_{12} therapy. Blood xylose levels were normal with the exception of the initial value in Case 1. Serum carotene was initially depressed in these same 2 patients but rose to low normal levels with treatment. Two of 3 patients had normal salivary iodide excretion times. The third had a delayed iodide appearance time which returned to normal on B_{12} therapy.

TABLE III. SERUM CAROTENE AND D-XYLOSE DETERMINATIONS IN PERNICIOUS ANEMIA PATIENTS IN RELAPSE

CASE	AGE	HEMATOCRIT	DATE	2 HOUR BLOOD XYLOSE (MG. PER CENT)	5 HOUR URINE XYLOSE (GM.)	SERUM CAROTENE (μG PER CENT)
1 J H	76	30	11/ 9/57	15.5	1.9	19.7
J H		38	11/18/57	39.7	0.4	36
J H		42	3/ 6/58	21.4	2.6	69
2 J S	75	30	1/ 3/58	32.2	0.8	27.5
J S		25	1/16/58	23.3	2.0	28.4
J S		42	3/ 6/58	40.0	4.1	45
3 M H	56	29	2/19/58	42.3	6.0	97
M H		37	3/16/58	42.1	6.0	115
4 M C	60	26	5/16/58	38.2	6.1	66
5 E C	72	26	5/15/58	43.1	4.2	92

Blood xylose levels in 6 patients with nonmegaloblastic anemia were normal (Table IV and Fig. 2). Urine xylose excretion was normal in 4 of this group and low in the remaining 2 (Table IV and Fig. 1). Serum carotene was within the normal range in 5 patients. One subject with Cooley's anemia had a value of 40 μg per cent.

TABLE IV. SERUM CAROTENE AND D-XYLOSE DETERMINATIONS IN PATIENTS WITH ANEMIA OTHER THAN PERNICIOUS ANEMIA

CASE	AGE	HEMATOCRIT	DIAGNOSIS	2-HOUR BLOOD XYLOSE (MG. PER CENT)	5-HOUR URINE XYLOSE (GM.)	SERUM CAROTENE (μG PER CENT)
1. A. P.	41	18	D.U.* with acute and chronic blood loss	14.2	2.2	72
2. A. T.	35	25	Cooley's anemia	22.5	5.4	40
3. J. E.	74	27	Gastric polyp with chronic blood loss	42.5	4.5	75
4. G. M.	19	30	Hepatosplenomegaly with normocytic anemia	18.5	7.0	59
5. B. G.	18	35	Chronic infection with normocytic anemia	43.8	5.8	56
6. E. D.	43	35	D.U.* with chronic blood loss	57.1	2.9	181

*Duodenal ulcer.

DISCUSSION

Fishberg and Friedfeld[15] in 1932 demonstrated diminished urinary excretion of D-xylose in the presence of renal insufficiency. Helmer and Fouts[14] evaluating small intestinal absorption, noted a marked decrease in urine xylose excretion over a 5-hour period in 3 patients with steatorrhea. The importance of determining blood xylose was emphasized, in order to help differentiate diminished intestinal absorption from impaired renal clearance. Fourmann[8] and Benson and associates[9] also reported low 5-hour urinary xylose values (1 to 2 Gm.) in the sprue syndrome. Although some xylose is actively metabolized in the human body,[13, 16] the test is considered useful clinically in screening patients for intestinal malabsorption.

Although Helmer and Fouts[14] found slightly impaired absorption of xylose in a few of their patients with pernicious anemia in remission, no consistent abnormality was observed. Kidney function, as measured by urea clearance, markedly influenced the amount of xylose in the urine. In individual cases, however, urine xylose excretion did not necessarily parallel the urea clearance. Of our 20 patients in remission, 13 presented normal values for blood and urine xylose. The remaining 7 excreted diminished amounts of xylose in the urine. The presence of an elevated nonprotein nitrogen in Case 1 and normal blood xylose levels in Cases 2 to 7 favor diminished renal excretion rather than impaired intestinal absorption, although a minimal degree of the latter cannot be ruled out.

When patients in relapse were treated with specific antianemic therapy by Helmer and Fouts,[14, 17] there was a rise in urea clearance and an increase in xylose excretion. We also found increasing urinary xylose excretion in

2 of our 5 patients undergoing hematologic remission. The 3 remaining patients, despite a comparable degree of anemia, had normal urinary xylose values.

There was no consistent impairment of xylose absorption in patients with a moderate degree of anemia other than pernicious anemia. The presence of an elevated blood xylose level with low urinary excretion in Case 6 suggests impaired renal clearance. The possibility that a profound degree of anemia may result in impaired xylose absorption is raised by Case 1, who with a hematocrit of 18 per cent, had low levels of both blood and urine xylose.

Wenger *and associates*[7] *have shown by fat balance studies that diminished* intestinal fat absorption correlates well with low serum carotene levels, values below 50 μg per cent being the rule. Elevated serum carotene may occur with dietary excesses of carotene containing foods, and is also found in hypothyroidism, diabetes mellitus, and hyperlipemia. Low levels are seen with deficient intake, fever, jaundice, celiac disease, sprue, and hyperthyroidism.[21] Rubegni,[19] in a study of 5 patients with pernicious anemia, reported serum carotene levels within the lower limits of normal unrelated to the hemoglobin concentration.

In the *present* study *all pernicious anemia patients in remission had normal* blood carotene levels. Three patients in relapse, with adequate dietary intake, presented low normal values. The remaining 2 patients with a history of anorexia, nausea, and vomiting showed low levels of serum carotene which rose progressively with therapeutic remission and resumption of normal food intake. Thus, reduced intake rather than impaired absorption would appear to explain these low values since it has been shown[18, 20, 22] that diet, the only source of body carotene, directly influences blood levels.

Five of 6 patients with anemia other than megaloblastic had carotene values within the normal range.

Excretion of salivary iodide following oral ingestion of potassium iodide was normal in 12 patients with pernicious anemia in remission in this study, whereas only 1 of 3 patients in relapse had a delay in excretion. These observations are at variance with those of Heath and Fullerton[10] who reported some delay in iodide excretion in 7 of 14 patients with pernicious anemia in remission. These differences may possibly be accounted for by variations in gastric emptying time, not measured in either study.

Clark[23] has shown that there is no abnormality of folic acid absorption in pernicious anemia.

SUMMARY AND CONCLUSIONS

No consistent impairment of intestinal absorption of D-xylose, carotene, or potassium iodide was demonstrated in patients with pernicious anemia in hematologic remission. Two of 5 patients in relapse showed low serum carotene levels and low levels of urinary xylose excretion in the presence of normal blood values. The increase in urine xylose content following therapy was presumed to be due, in part, to increased renal clearance associated with a rising hematocrit. Resumption of normal eating habits was associated with a rise of serum carotene to low normal levels.

Although the possibility of minimal malabsorption of substances other than vitamin B_{12} exists in patients with pernicious anemia, particularly during relapse, the degree of malabsorption does not appear to be significant.

We wish to thank Dr. Bernard Cooper of the Thorndike Memorial Laboratory, Boston City Hospital, for his assistance in selecting the patients for this study.

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STUDIES IN LEUKEMIA

IX The Induction of Leukemia in Adult C3H Mice by Means of Cell-Free Brain Filtrates

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THE opportunity to extend our studies[1-3] on the induction of leukemia with cell free brain filtrates presented itself when a spontaneous leukemia developed in a 22-week-old C3Heb male mouse in our breeding colony

MATERIALS AND METHODS

Preparation of Cell Free Filtrates—The cell free filtrates of brain are prepared in the following manner:

1 Half of the mouse brain is removed immediately after death and placed in sterile saline solution in a 10 by 75 mm test tube When the brain is not to be processed immediately the tube is stoppered with a cotton plug and stored at –15° C

2 Mortar and pestle for grinding, a 100 ml graduated cylinder, and a flask are kept cold in the freezer

3 An ice bath consisting of a pan filled with ice cubes and ice water is kept at 4° C along with a flask of buffered isotonic saline solution of pH 7.4

4 The cold mortar is placed in the ice bath The brain is ground in the mortar with a small amount of saline solution and some sand This is done in a cold room or in the refrigerator It takes about 10 minutes until only a fine suspension remains, which is poured into the graduate, the mortar is rinsed with the cold buffered saline solution in order to dilute the brain suspension to 100 ml

5 The suspension is then transferred to a flask where it is left to stand in the refrigerator for 24 hours This step does not seem critical, however, because many filtrates have been used after settling for only an hour

6 If after standing the supernatant is relatively clear, it may be decanted If the supernatant is very cloudy, it is centrifuged at 3,000 r p m for 15 minutes at 4° C

7 Just before filtration the supernatant is contaminated with *Esch coli*

8 The contaminated supernatant is filtered through a Seitz so called sterilizing pad (Type S)

9 A sample specimen is taken for bacterial culture before and after filtration

10 Either the filtrate is used immediately or it is stored until needed in sterile cotton plugged tubes at –15° C

11 For intraperitoneal inoculations, 0.5 ml of the filtrate is used For intracerebral inoculations, from 0.075 to 0.1 ml is used

12 Recipients are usually 4 to 12 week old mice

From the Hektoen Institute for Medical Research of the Cook County Hospital Chicago Ill

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Preparation of Tumor Cell Suspensions.—Tumor cell suspensions are prepared by homogenizing tumor in a glass tissue mill and diluting the cells with physiologic saline solution to a concentration of approximately 10^6 per cubic centimeter. Tumor cell inoculations are made intraperitoneally with 0.5 ml. of the cell suspension.

Source of Mice.—The C3Heb mice were obtained either directly from Jackson Memorial Laboratory or were bred from a pedigreed stock originally obtained from them. The C3H mice were from Jackson Memorial Laboratory or from Cumberland View Farm, Clinton, Tenn. The C3H (Heston) mice were obtained from Dr. Sarah Stewart, National Institute of Health. The C3H x 101 hybrid mice were from Cumberland View Farm, Clinton, Tenn. The C3H (Rockland) mice were obtained from Rockland Farms, New City, N. Y.

RESULTS

Characteristics of Spontaneous Leukemia.—The 22-week-old male C3Heb mouse which served as the springboard for these experiments was 1 of 12 C3Heb mice in which spontaneous leukemia developed in our colony. Nine of the 12 were female, 3 were male. Their ages ranged from 22 to 91 weeks (average 50.4 weeks). Four of the animals were closely related. One female and one male were litter mates; the female had 3 litters; 2 of the mice of the third litter also developed leukemia. None of the female progeny had subsequent litters; thus, this line could not be studied further.

The spontaneous leukemias were characterized by a generalized lymphadenopathy, including a discrete mesenteric tumor, enlargement of the thymus, and moderate hepatosplenomegaly. The blood and marrow were studied in 4 of the spontaneous leukemias. Of the white cells in the blood, from 5 to 95 per cent were primitive lymphoid cells. When the percentage was low, the lymphocytes were increased in number; many showed bilobed and bizarrely-shaped nuclei; the cytoplasm frequently took a deep basophilic strain. The marrow was partly or completely replaced by the lymphoid cells. These varied in size from 12 to 16 micra and had a deep basophilic cytoplasm. The nuclei showed distinct pleomorphism and usually occupied about four-fifths of the cell; the chromatin was coarse and one or more nucleoli were frequently present.

The histologic changes in these animals resembled those observed in the lymphoblastoma of AKR mice.

Attempts were made to pass two of the other spontaneous leukemias with both tumor cell suspensions and brain filtrates. Those attempts were unsuccessful in 8 C3H and 31 C3Heb mice.

There were a total of 1,538 C3Heb mice over 22 weeks of age in our breeding colony which had never been used for experimental purposes. Those included 827 females and 711 males. This gives an incidence of 1.1 per cent in females and 0.4 per cent in males, for an over-all incidence of 0.6 per cent. This is similar to the incidence of 3 in 545 (0.6 per cent) females and 1 in 273 (0.4 per cent) males in Dr. Elizabeth Fekete's studies at Jackson Memorial Laboratory.[4]

Transmission by Means of Tumor Cell Suspensions.—The data for the transmission of the C3H lymphoma by means of tumor cell suspensions are summarized in Table I.

TABLE I Results of Intraperitoneal Inoculation of C3H Mice With Tumor Cell Suspensions

GROUP	RECIPIENT STRAINS	SEX	RANGE OF SURVIVAL (DAYS)	NO EXPERIMENTS	NO LEUKEMIC / NO INJECTED	PER CENT LEUKEMIC
1	C3Heb (Jax)	F	10 17	7	39/40	97
2	C3Heb (Jax)	M	9 21	10	56/59	95
3	C3H (Jax)	F	10 18*	7	31/31†	100
4	C3H (Jax)	M	13 19	3	22/25	88
5	C3H (Clinton)	F	10 15	6	37/39	95
6	C3H (Clinton)	M	13 15	1	9/10	90
7	C3H (Heston)	F	9 14	5	31/35	88
8	C3H (Heston)	M	10 22	3	14/14	100
9	C3H (Rockland)	F	17 21	2	4/20	20
10	C3H (Rockland)	M	16 21	1	4/10	40
11	C3H x 101 (Clinton)	F	13 18	4	19/24	79
12	C3H x 101 (Clinton)	M	14 20	4	27/34	79
				53	293/341	86

*One animal survived 35 days

†Five animals (in one experiment) that died of infection within the first 3 days after inoculation are not included

1 In Group 7 all animals succumbed to the transplanted lymphoblastoma in 4 of the experimental groups In one group, all 4 animals survived the tumor cell inoculation

2 In Groups 11 and 12, 12 mice died of a salmonella infection In all others lymphoblastoma developed

3 Significant differences are not apparent between the susceptibility of the sexes, or the substrains, with the exception of the C3H (Rockland) mice (Groups 9 and 10) Although only a few experiments with these animals have been done, it appears that their susceptibilty to this tumor is far less than that of the other substrains

4 Data do not indicate any significant difference between isologous and homologous passage of the tumor Of the 53 experiments, 21 were made within the substrain, and 32 were across substrain lines Neither the incidence nor the latency is influenced by the source of the tumor, or by continuous passage One tumor line has been maintained through 14 passages Leukemia developed in 9 out of 10 mice in 17 to 20 days in the first passage, and developed in 10 out of 10 in 13 to 18 days in the fourteenth passage

Transmission by Means of Cell-Free Brain Filtrates—The data for the transmission of the C3H lymphoma by cell free brain filtrates are summarized in Table II

1 There is no significant difference in either incidence or latency with regard to the sex of the recipient

2 The low incidence of leukemia in Group 3 is accounted for in part by the fact that 6 animals of one group died within 48 hours after injection Interestingly, however, in none of the 5 mice of the other group in this same experiment did leukemia develop when the filtrate of a C3H (Jax) brain was used

for filtrate source. This same filtrate failed to produce leukemia in any of 10 C3Heb (Jax) mice when inoculated intraperitoneally (Group 5). In one other experiment, a filtrate prepared from the brain of a C3H (Heston) mouse failed to induce leukemia in any of 5 female C3H (Heston) mice inoculated intraperitoneally (Group 13).

TABLE II. RESULTS OF INOCULATION OF C3H MICE WITH CELL-FREE BRAIN FILTRATES

GROUP	RECIPIENT STRAINS	SEX	ROUTE INOCUL.	RANGE (DAYS) SURVIVAL	NO. EXPERIMENTS	NO. LEUKEMIC / NO. INJECTED	PER CENT LEUKEMIC
1	C3Heb (Hektoen)	F	I.C.	14-29	3	12/13	92
2	C3Heb (Hektoen)	M	I.C.	14-29	3	16/16	100
3	C3Heb (Jax)	F	I.C.	13-17	2	4/15*	27
4	C3Heb (Jax)	M	I.C.	14-25	5	39/42	93
5	C3Heb (Jax)	F	I.P.	9-17	4	23/34	67
6	C3Heb (Jax)	M	I.P.	14-20	1	9/10	90
7	C3H (Jax)	F	I.C.	12-20	4	29/29	100
8	C3H (Jax)	F	I.P.	13-28	3	15/15	100
9	C3H (Jax)	M	I.P.	22-27	1	5/5	100
10	C3H (Clinton)	F	I.C.	12-18	1	14/15	93
11	C3H (Clinton)	F	I.P.	10-15	2	23/25	92
12	C3H (Clinton)	M	I.P.	12-21	3	38/40	95
13	C3H (Heston)	F	I.P.	11-15	3	9/14	65
14	C3H (Heston)	M	I.P.	10-19	2	9/9	100
15	C3H (Rockland)	F	I.P.	38-46	1	2/10	20
16	C3H (Rockland)	M	I.P.	10-37	2	11/20	55
17	C3H x 101 (Clinton)	F	I.P.	13-29	5	35/39	89
18	C3H x 101 (Clinton)	M	I.P.	13-29	3	16/27	60
					48	309/378	82

I.C. = intracerebral.
I.P. = intraperitoneal.
*Six animals that died within 48 hours of inoculation are included.

As in the passages with tumor cell suspensions, the significantly lower incidence in C3H (Rockland) mice (Groups 15 and 16) should be observed. The latent period for the development of leukemia in C3H (Rockland) females also seems longer than in any of the other mice, and although the males of this strain also show some delay in the development of leukemia, the degree is much less striking.

3. Significant differences were not demonstrable in the results regardless of isologous or homologous brain filtrates. Of the 48 experiments, 27 were within the substrains and 21 were across substrains. Continuous passages, up to 5, did not influence either the latency or incidence of the development of leukemia. In 15 of the 48 experiments the filtrates were prepared from the brains of mice in which leukemia developed as the result of the inoculation with tumor cell suspensions. Of the 141 mice inoculated with these filtrates, leukemia developed in 120 (85 per cent). This was similar to the incidence in the other 33 experiments, indicating that there is no significant difference between the various sources of the brain filtrates regarding their ability to produce leukemia.

4. In addition to the animals shown in Table II, 14 animals were injected subcutaneously with cell-free filtrates of brain. Ten of these were less than 24 hours old at the time of injection. In all 10 of these mice subcutaneous tumors developed at the injection site (Fig. 1); moreover, disseminated disease occurred, and the animals died in from 20 to 50 days after injection. The remaining 4 animals were 1 week of age at the time of inoculation. In 2, leukemia developed in 20 and 26 days.

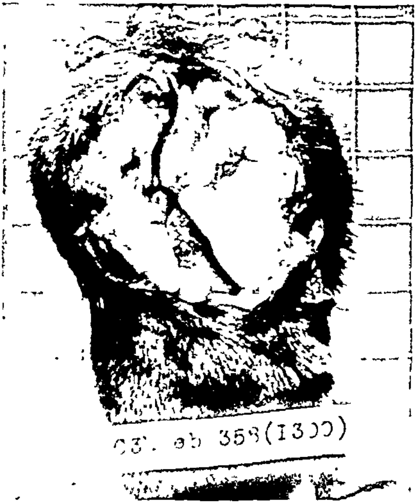

Fig. 1.—Fifty-day-old C3Heb mouse injected as newborn with cell-free brain filtrate Massive subcutaneous lymphosarcoma at site of injection

5. Twenty mice were injected with a cell-free brain filtrate which had been exposed to 100,000 r delivered by the Telecobalt^{60} machine Leukemia developed in 11 of these mice.

TABLE III. RESULTS OF INOCULATION OF C3H MICE WITH HEAT INACTIVATED BRAIN FILTRATES

SOURCE OF FILTRATE	RECIPIENT STRAINS	ROUTE OF INJECTION	UNHEATED NO LEUKEMIC / NO INJECTED	HEATED NO LEUKEMIC / NO INJECTED
C3H (Jax)	C3H (Jax)	I.P.	15/15	0/10
C3H (Jax)	C3H (Jax)	I P	15/15	0/10
C3Heb (Jax)	C3H (Clinton)	I.C	14/15	0/8
C3H (Clinton)	C3H (Clinton)	I.P.	10/10	0/10

The Effect of Inoculation of Control Brain Filtrates Into C3H Mice.—The results with heat-inactivated (65° C. for 45 min.) leukemic brain filtrates are summarized in Table III. Female mice were used in these experiments. It is apparent that heat inactivates the filtrates.

The results of inoculating C3H mice with cell-free brain filtrates prepared from nonleukemic C3H mice are summarized in Table IV. Leukemia did not develop in any of the mice so treated.

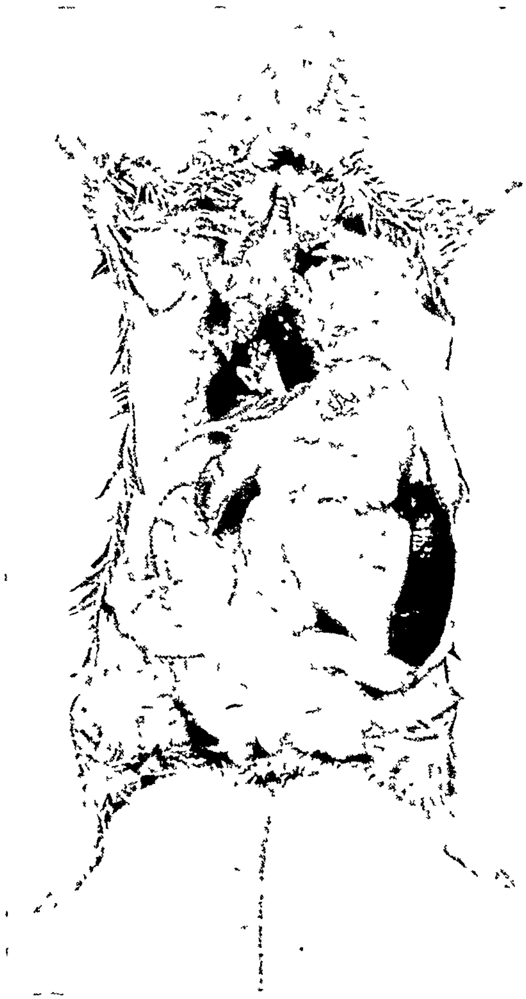

Fig 2—Adult C3H mouse, 14 days after intracerebral inoculation of cell-free brain filtrate Note massive diffuse mesenteric involvement with invasion of kidneys and genital organs

Pathologic Changes.—The gross appearance of the induced leukemia is illustrated in Fig. 2. The characteristic is a massive, diffuse infiltration of the mesentery with involvement of the urogenital organs, the involvement frequently extending through the peritoneum to infiltrate the abdominal and perispinal musculature Involvement of the liver and spleen varies from slight to massive Peripheral lymph node involvement is usually only moderate, and thymic involvement is modest

TABLE IV RESULTS OF INOCULATION OF C3H MICE WITH NONLEUKEMIC BRAIN FILTRATES

SOURCE OF FILTRATE	RECIPIENT STRAIN	SEX	ROUTE OF INJECTION	NO EXPERIMENT	NO LEUKEMIC / NO INJECTED
C3H (Jax)	C3H (Jax)	F	I P	2	0/16
C3H (Jax)	C3H (Clinton)	M	I P	2	0/15
C3Heb (Jax)	C3H (Clinton)	F	I C	2	0/15
C3H (Clinton)	C3H (Clinton)	F	I P	1	0/10

The histologic changes are typical of mouse lymphoblastoma and are indistinguishable from those described previously regarding Swiss mice[3]

Among the 25 animals whose blood was studied, the presence in 9 of at least 5 per cent primitive lymphoid cells was considered diagnostic of "leukemia" Whenever the blood was considered diagnostic, the marrow was always frankly involved Of the 6 animals whose blood was not considered diagnostically characteristic, *diffuse or massive infiltration by tumor cells was present in the* marrow

DISCUSSION

The induction of leukemia in C3H mice by means of cell free brain filtrates adds further evidence to the thesis that leukemia may be induced by an agent present in cell free extracts of brain The characteristics of this agent with regard to resistance to roentgen ray, heat inactivation, and filterability are comparable to those previously described for the filtrates prepared from the brains of AKR[1] and Swiss[3] mice and from the brains of patients dying of acute leukemia[2]

As with the other filtrates, the percentage of animals in which leukemia developed after inoculation is high, and tends to approach an *all* or none response Occasionally, leukemia fails to develop in whole groups of animals when inoculated with a brain filtrate Resistance in these animals represents a phenomenon different from that observed in C3H (Rockland) mice in which the incidence of leukemia is low as induced with both tumor and filtrate In the case of filtrate induced leukemia, the substrain seems to have a specific resistance to the leukemia In tumor induced leukemia, there is no resistance to tumor cell inoculation, resistance to *filtrate inoculation usually occurs in whole groups of* animals inoculated with the same filtrate For this reason it appears that these negative experiments result from a function of the filtrate rather than of the host

The induction of leukemia by a cell free brain filtrate compels the question regarding the mechanics of leukemogenesis Pathogenic studies[5] demonstrate the multicentric origin of this disease They suggest that the pluripotential mesenchymal cells are capable of undergoing leukemic transformation anywhere in the body

We believe that the cell-free brain filtrate is rich in the virus which, whether injected intracerebrally or intraperitoneally, becomes rapidly disseminated, and begins to multiply. The mesenchymal cell proliferation characteristic of this virus multiplication is a defensive host reaction. In the newborn animal, the more primitive and energetic mesenchymal host reaction is capable of producing subcutaneous tumors at the site of injection in addition to producing a disseminated disease.

SUMMARY

1. Spontaneous leukemia developed in a 22-week-old male C3Heb mouse.

2. Tumor cell suspensions obtained from the original leukemic mouse or from other mice to which leukemia was transmitted when injected into various C3H substrains and C3H x 101 hybrids caused leukemia in 293 of 341 mice (86 per cent).

3. Cell-free brain filtrates, similarly obtained, inoculated into the same substrains induced leukemia in 309 of 373 mice (82 per cent).

4. The experiments further substantiate the thesis that leukemia may be induced in the mouse by a cell-free agent having the characteristics of a virus.

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SEROLOGIC REACTIONS IN ADENOVIRUS DISEASE

I. Delayed Complement-Fixing Antibody Response

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During the past several years there has been steadily increasing evidence to implicate certain members of the adenovirus family as important in the causation of respiratory disease in military populations. The syndromes with which the implicated virus types have been associated cover a spectrum that ranges from relatively simple upper respiratory infections to atypical pneumonias of varying severity.[1-6]

Laboratory techniques applicable to the etiologic diagnosis of adenovirus disease include virus isolation, neutralization tests, and complement fixation tests.[1, 2, 6, 7] The first two methods are quite cumbersome and even the colorimetric modification of the neutralization test described by Lennette and associates[8] is relatively time consuming and must be rigidly controlled. Furthermore, isolation of an adenovirus from a patient does not necessarily prove its causal relationship to the illness.

Complement fixation tests, on the other hand, afford a fairly simple means of detecting adenovirus infections, and the relative simplicity of the technique permits its application to fairly large groups of individuals. Although the method does not identify the adenovirus serotype responsible for the infection the technique can be utilized with a considerable degree of accuracy in epidemiologic studies, particularly when ancillary knowledge is available as to the serotypes that are operating in the population under study. In serologic tests for the diagnosis of viral infections, and including those for the adenoviruses, a diagnostically significant finding is considered to be the occurrence of a fourfold or greater rise in antibody titer during the course of the illness. The detection of increases in antibody is based on the examination of multiple serum specimens. The first specimen is obtained early in the course of the illness, and the second specimen is usually obtained about two weeks after the first. It has been our experience that this generalization is applicable to the study of adenovirus infections, but exceptions have been encountered and form the basis of the present report. In this connection it is noteworthy that

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the patient from whom the prototype RI-67 strain of type 4 adenovirus was isolated did not show a rise in complement-fixing antibody level at 14 days after onset of illness, but a rise in antibody titer was detected at a later time.[1]

MATERIALS AND METHODS

Collection of Serum Specimens and Throat Washings.—The blood samples and throat washings examined in this study were collected in the course of a more comprehensive study of respiratory disease conducted at Fort Ord, Calif., from September, 1954, through August, 1955. A part of that major study was concerned with the proportion of patients admitted to the Station Hospital with respiratory disease attributable to infection with an adenovirus.

For purposes extraneous to this report, 3 blood samples were obtained wherever feasible from men admitted to the respiratory disease wards of the Station Hospital. Feasibility depended on the individual's remaining on-post sufficiently long to permit collection of the second and third blood samples. The first specimen (acute phase) was obtained as soon as possible after the onset of illness; the second (convalescent phase) was obtained, insofar as possible, 14 days thereafter; and the third specimen (late convalescent phase) was taken 14 or more days after the second.

Throat washings were not obtained from every patient because of the technical burden posed by the collection and testing of such a huge volume of material. Instead, washings were collected from a cross-section of the population, namely, from 434 of the 3,060 patients admitted to the study; the specimens were obtained at the time the first blood sample was taken.

Laboratory Tests Employed.—These consisted of the complement fixation and neutralization tests and virus isolations.

Complement fixation tests were done according to the technique employed in this laboratory for some years and described in a recent compendium on diagnostic methodology.[9] The antigen consisted of HeLa cell cultures infected with the RI-67 strain of type 4 adenovirus. Sets of serum samples (first and second, or first and third specimens) from the same individual were always examined simultaneously in the same tests.

Neutralization tests were done by the colorimetric technique described in another communication from this laboratory.[8] Acute and late convalescent phase serum specimens from the same individual were always examined simultaneously in the same test. Three adenovirus serotoypes, viz., 3, 4, and 7, were used. The type 3 strain was the GB strain, the type 4 was the RI-67 strain, and the type 7 was the Gomen strain.

Virus isolation was based on the use of HeLa cell culture systems. Identification and typing of cytopathogenic agents as adenoviruses were done by the colorimetric method,[8] and monotypic antisera (rabbit) were employed.

RESULTS

During the period of this study, adenoviruses were recovered from 129 of the 434 individuals from whom throat washings were collected and tested. Subsequent typing of these 129 adenoviruses showed the following distribution of serotypes: 1 of type 3, 85 of type 4, and 43 of type 7.

The multiple serum specimens from those individuals from whom an adenovirus had been isolated were examined for their complement-fixing and neutralizing antibody levels, and the findings evaluated against virus isolation as a base line.

Delay in the Appearance of Complement-Fixing Antibody.—Collation of the results of the complement fixation tests with the results of virus isolation tests revealed that in 120 of the 129 patients from whom an adenovirus was isolated, a fourfold or greater rise in antibody titer occurred during the approximate 2-week interval between the drawing of the acute phase blood specimen and the early convalescent phase blood specimen. Stated in other terms, a *diagnostically* significant rise in complement-fixing antibody titer was encountered in 93 per cent of the patients from whom an adenovirus was recovered. The remaining 9 individuals from whom an adenovirus had been isolated showed no rise in complement-fixing antibody titer in the interval between the acute and early convalescent phase bleedings. The findings on these 9 patients are presented in Table I. It will be noted that the acute phase blood specimens had been obtained within 5 days of the onset of illness, and that the early convalescent phase specimens had been obtained from 14 to 20 days after onset. The absence of antibody at these relatively late periods after onset of illness bore no obvious relation to the serotype of the virus recovered, inasmuch as 1 patient yielded a type 3 virus, 5 patients yielded a type 4, and 3 patients yielded a type 7 virus. These cases were not restricted to any one temporal period, but occurred throughout the study year and were intermingled with a much larger number of patients from whom an adenovirus was isolated and who showed diagnostically significant rises in complement-fixing antibody titer.

TABLE I. ABSENCE, OR DELAYED APPEARANCE, OF COMPLEMENT-FIXING ANTIBODY IN ADENOVIRUS DISEASE

SUBJECT NUMBER	VIRUS ISOLATED (SEROTYPE)	ACUTE PHASE BLOOD		CONVALESCENT PHASE BLOOD		LATE CONVALESCENT PHASE BLOOD	
		DAYS FROM ONSET	C F TITER	DAYS FROM ONSET	C F TITER	DAYS FROM ONSET	C F TITER
1	3	3	<1:8 <1:8	18	<1:8	31	1:64
2	4	4	<1:8 1:8	17	<1:8	43	1:256
3	4	3	<1:8 <1:8	14	<1:8	33	1:32
4	4	4	<1:8 <1:8	18	<1:8	38	<1:8
5	4	3	<1:8 1:8*	17	1:8	37	1:64
6	4	4	<1:8 <1:8	20	1:8	36	1:32
7	7	1	<1:8 <1:8	15	<1:8	29	1:16
8	7	5	<1:8 <1:8	19	<1:8	36	<1:8
9	7	2	<1:8 <1:8	18	<1:8	49	1:16

*Nonspecific fixation.

The tests on these serologically negative patients were repeated using the acute phase blood specimen and a third, or late convalescent, specimen, the latter specimens had been taken from 29 to 49 days after the onset of the respiratory illness (Table I). As is shown in Table I, in 7 of the 9 patients a significant rise in complement-fixing antibody had occurred at some time during the interval between the second and third bleedings, i.e., between the time the early convalescent and the late convalescent blood specimens had been taken. In two instances (Subjects 4 and 8), the isolation of an adenovirus was not accompanied by the appearance of detectable amounts of complement-fixing antibody, even with blood specimens taken as late as 36 and 38 days after the onset of the illness.

Relationship Between Virus Isolation and Neutralizing Antibody Titers.—To determine whether the situation with respect to neutralizing antibody paralleled or differed from that observed with the complement-fixing antibody, neutralizing antibody levels were determined in the sera of those 9 individuals described above and listed in Table I. Unfortunately, by this time the early convalescent phase (2 to 3 weeks) serum specimens were no longer available, so that antibody determinations were performed on the acute phase and late convalescent phase blood specimens. As is shown in Table II, a fourfold or greater rise in neutralizing antibody level was demonstrable in all 9 individuals, including the 2 patients in whom no detectable complement-fixing antibody response was observed.

TABLE II. NEUTRALIZING ANTIBODY RESPONSE IN PATIENTS IN WHOM COMPLEMENT-FIXING ANTIBODY RESPONSE TO ADENOVIRUS INFECTION WAS DELAYED OR NONDEMONSTRABLE

		ANTIBODY TITER TO					
		TYPE 3 VIRUS		TYPE 4 VIRUS		TYPE 7 VIRUS	
SUBJECT NUMBER	VIRUS ISOLATED (SEROTYPE)	ACUTE PHASE BLOOD	LATE CONV. PHASE BLOOD	ACUTE PHASE BLOOD	LATE CONV. PHASE BLOOD	ACUTE PHASE BLOOD	LATE CONV. PHASE BLOOD
1	3	<1:4	1:16	<1:4	<1:4	<1:4	<1:4
2	4	<1:4	<1:4	<1:4	1:16	<1:4	<1:4
3	4	<1:4	<1:4	<1:4	1:32	<1:4	<1:4
4	4	<1:4	1:16	<1:4	1:8	<1:4	1:8
5	4	<1:4	1:8	<1:4	1:16	<1:4	<1:4
6	4	1:4	1:4	<1:4	1:16	<1:4	<1:4
7	7	<1:4	1:8	<1:4	1:16	<1:4	1:4
8	7	<1:4	1:32	<1:4	1:8	<1:4	<1:4
9	7	<1:4	1:4	<1:4	1:16	<1:4	<1:4

A point of interest was the heterotypic nature of the neutralizing antibody response. In 4 patients (Subjects 1, 2, 3, and 6) the neutralizing antibody response was homotypic, and there was no evidence of an antibody response to a heterotype. In 4 other individuals (Subjects 4, 7, 8, and 9), the heterotypic antibody response was greater than the response to the patients' own infecting virus, i.e., the homotypic response. In a fifth patient (Subject 5), who showed a rise in heterotypic antibody, the rise was of a slightly lesser magnitude than the rise in homotypic antibody. A surprising feature was the failure of all 3 individuals from whom a type 7 virus was isolated to develop neutralizing antibody to this serotype; all 3 individuals responded, rather, with significant increases in type 4 antibody, and 2 of the 3 also showed diagnostically significant increases in type 3 antibody levels. The number of individuals (4 of 8) who responded heterotypically with type 3 antibody is quite high and appears disproportionate in view of the fact that a type 3 virus was encountered only once among 129 adenovirus isolations It is also of interest that the greatest heterotypic increases in type 3 neutralizing antibody occurred in the 2 patients (4 and 8) who failed to show demonstrable amounts of complement fixing antibody in their blood even one month after the onset of illness

DISCUSSION

The data presented do not impugn the validity of studies using complement fixation tests as the primary tool in the diagnosis of adenovirus disease in adults The lack of correlation between the isolation of an adenovirus and the appearance of a complement fixing antibody response within 2 to 3 weeks after onset of illness occurred in only 5 per cent of the 129 cases Under some circumstances, however, such discrepancies might assume some importance It should be pointed out, however, that the results reported in this paper were derived from studies during one year on a single, rather homogeneous population of young men, thus it is not inconceivable that under some circumstances, as in a population group not previously exposed to infection, a greater lack of correlation might be encountered

Prior to the completion of this report the paper of Katz and associates[10] described a disassociation between neutralizing and complement fixing antibody levels Their studies dealt with adenovirus types 1 to 4 and were concerned with individuals who could not be considered to be suffering from adenovirus disease, but who had residual antibody They comment that the lack of consistent relationship between neutralizing and complement fixing antibody deserves further consideration in relation to its significance in adenovirus infections and adenovirus disease From one point of view, namely, the practicality of using complement fixation tests in the serodiagnosis of adenovirus disease, the data presented herein afford a partial answer Nonetheless, it must be remembered that this study was carried out in a circumscribed group and that the results might be quite different under other conditions Differences might also be observed with other adenovirus types

There is general agreement that adenovirus types 3, 4 and 7 are, *at present,* the most prominent causes of adenovirus disease in military populations in the United States (see précis in van der Veen and Kok[11]) It is becoming clearer that *adenovirus disease due to type 3 virus is,* at least currently of the least proportionate importance The limited body of data presented herein suggest there is a tendency for infections with type 4 or type 7 viruses to elicit a heterotypic antibody response to type 3 virus This finding agrees with the observations of Hilleman and associates[12] who noted that inoculation with vaccines containing only type 4 and type 7 adenoviruses called forth an *appreciable rise in antibody to type 3 virus* Both observations thus afford additional evidence for the concept that disease due to type 3 virus is relatively less important, incidence wise, in this age group (recruits) because of previous experience

SUMMARY

In young men with respiratory disease there was usually good correspondence between the isolation of an adenovirus and the appearance of an increase in complement-fixing antibody in about 2 to 3 weeks. In 9 of the 129 cases, an increase in complement-fixing antibody in this period of time was not demonstrable, but in 7 of these 9 instances a definitive increase in antibody titer could be found after 29 or more days. In 2 of the 9 cases, complement-fixing antibody failed to rise even at this late time.

When the acute and late convalescent phase sera were assayed for neutralizing antibody, a rise in titer was detected in all 9 individuals in whom demonstrable complement-fixing antibody either failed to appear or was delayed in appearing. The neutralizing antibody response was often heterotypic, however, bearing no relationship to the virus serotype isolated from the individual.

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MIXTURES OF COAGULASE POSITIVE STAPHYLOCOCCI AND STRAIN VARIATION IN SPECIMENS FROM CLINICAL SOURCES

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THE recognition of staphylococcal strains either as discrete in origin or as variants of a common parental strain has been greatly simplified in recent years by the development and adoption of bacteriophage typing methods[1 3] The occurrence of mixed growth of staphylococci would be expected from the ubiquitous distribution and large numbers of known types There is a common tendency, however, to treat clinical specimens as containing only a single strain of a given pathogen or potentially pathogenic species In the course of routine bacteriophage typing of staphylococci it was learned that a specimen so treated and recorded as nontypable, actually proved to be a mixture of two typable strains with complementary patterns of phage sensitivity This observation was expanded into the following study of the incidence of mixtures of coagulase positive staphylococci in routine clinical specimens Staphylococcus Medium 110 was found to markedly stimulate pigment development, pigment variation, therefore, was employed in the presumptive detection of mixtures, followed by confirmatory tests of bacteriophage typing and antibiotic sensitivity testing on the isolated strains

METHODS

Routine clinical specimens received by the bacteriology laboratory at the Parkland Memorial Hospital were streaked on blood agar plates Confluent growth from plates containing coagulase positive staphylococci was transferred to trypticase soy agar slants for 4 to 6 hours at 37° C for enrichment and then streaked on plates of Staphylococcus Medium 110 These were incubated 48 hours at 30° C and an additional 48 to 72 hours at room temperature (25° C) Plates were examined daily after the second day for mixtures differing in pigmentation Only mixtures in which both components were coagulase positive by the slide coagulase test were further examined for antibiotic sensitivities and typed with bacteriophages

The bacteriophage typing methods of Blair and Carr[2] were employed for typing the strains with a set of 25 typing phages kindly supplied by Dr Blair

The resistance of each strain to 10 antibiotics and to triple sulfa was determined with the use of sensitivity disks (Baltimore Biological Laboratory)

RESULTS

A total of 196 clinical specimens containing coagulase positive staphylococci was screened for mixtures based on pigmentation Sixteen coagulase positive mixtures were obtained, ranging from pigmented plus white strains, readily detected by the first inspection, to pigmented mixtures which did not differ in color intensity until the fourth day Most of the pigmented mixtures would not have been detected on the original blood agar plates

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TABLE I. MIXTURES OF COAGULASE-POSITIVE STAPHYLOCOCCI FROM CLINICAL SPECIMENS

SPECIMEN NO.	SOURCE	PIGMENT	PHAGE PATTERN	DIFFERENCES IN ANTIBIOTIC PATTERN*
1	Ear infection	Light orange	47/53/77/VA4/42D	TC, AM, TM, SM
		Dark orange	73	
2	Throat swab	Light orange	42D/44A	
		Dark orange	3A	PC
3	Meningitis	Light orange	Nontypable	
		Dark orange	Nontypable	
4	Postoperative infection	Light orange	7/47/54/70/73/75/44A	
		Dark orange	Nontypable	AM, EM
5	Tracheal aspiration	Light orange	44A	
		Dark orange	44A	
6	Postoperative infection (?)	Orange	Nontypable	SSS
		White	52/52A/7/44A	
7	Lung (autopsy)	Light orange	7/47/53/54/73/75/77/VA4/44A	
		Dark orange	Nontypable	AM, EM
8	Tracheal aspiration	Light orange	44A	TC, AM, TM, PC, SM
		Dark orange	7/47/53/54/73/75	

9	Burn infection	Light orange	47/53/VA4	
		Dark orange	Nontypable	AM, EM
10	Postoperative infection	Light orange	79/7/47/53/54/73/77/VA4/44A	TC, AM, TM
		Dark orange	53	
11	Wound infection	Light orange	77	TC, AM, TM
		Dark orange	Nontypable	SSS
12	Sputum	Orange	Nontypable	
		White	44A	PC
13	Sputum	Orange	Nontypable	
		White	44A	PC
14	Infected umbilicus	Light orange	7/47/54/73/75	TC, AM, TM, PC, SM, SSS
		Dark orange	44A	
15	Throat swab	Orange	3A/44A	
		White	Nontypable	TC, AM, TM, BA, PC
15a	Sputum	Orange	3A/44A	
		White	Nontypable	TC, AM, TM, BA, PC
16	Ulcer	Light orange	Nontypable	TM
		Dark orange	Nontypable	

*Only differences are reported and symbol is placed on line with the resistant strain. TC = tetracycline, AM = chlortetracycline TM = oxytetracycline; SM = streptomycin; EM = erythromycin BA = bacitracin, SSS = triple sulfa, PC = penicillin.

Mixtures containing a coagulase-negative component occurred frequently but were not included.

Table I summarizes the results of bacteriophage typing and antibiotic sensitivity testing of the individual strains from the mixtures. Two specimens (3 and 5) probably represent color variants since the members of each mixture differ only in the property of pigmentation. All other specimens are presumably mixtures of two distinct strains, and all but one (16) are composed of either two bacteriophage types or a typable and nontypable strain. Since one-third of all the strains studied were nontypable, a mixture of nontypable strains would be expected; therefore, Strain 16 was considered a probable mixture on the basis of differences in antibiotic sensitivity and pigment. Two specimens (15 and 15a) are identical mixtures from the same patient and are regarded as one mixture. Mixtures of two coagulase-positive strains were therefore obtained from 14 individuals, or 7 per cent of the total number of specimens.

Several of the bacteriophage patterns are typical of the hospital strains of staphylococci and are probably hospital-acquired. In one individual (4) infection with the typable component is of a transient nature, since specimens taken before and after the mixture appeared were pure cultures of a nontypable strain. In another individual (3), however, strain 44A replaced a previously isolated nontypable strain.

All of the strains from the mixtures were sensitive to neomycin and carbomycin and resistant to polymyxin B. Variable results were obtained with the other antibiotics. For simplicity, only differences in sensitivity are recorded in the table, and it can be seen that in almost all mixtures one strain is generally more resistant than the other to one or more antibiotics.

TABLE II. VARIATION OBSERVED IN 31 CLINICAL SPECIMENS CONTAINING STAPHYLOCOCCI OF TYPE 80/81

PHAGE PATTERN	NUMBER OF SPECIMENS	
	PURE STRAINS	MIXTURES OF COLOR VARIANTS
A. 52/42B/81	5	
B. 52/42B/44A/81	20	2
C. Mixture of A and B		3
D. Mixture of 52/52A/42B/44A/81 and 52/52A/42B/81		1
Total	25	6

From these results it would seem that pigment variation within a strain is not common, having occurred in only two instances in the above series. However, we have recently observed that clinical specimens containing staphylococci with bacteriophage pattern 52/42B/81 (type 80/81[4]) included a number of light- and dark-pigmented variants. In a hospital outbreak caused by this type, a series of 31 specimens, primarily nasal swabs, were positive for the epidemic staphylococcal strain. Six of the specimens were mixtures of pigment variants and, of these, 4 also varied in bacteriophage typing pattern (Table II).

Variation in sensitivity to bacteriophage 44A has been previously reported for this staphylococcal type.[4, 5] The strain sensitive to bacteriophage 52A may differ from the type 80/81, although by the criteria of Williams and Rippon[1] they would be considered the same strain. There is some indication, however, that isolates with a typing pattern 52/52A/42B/81 are not as virulent as typical strains of type 80/81.[6]

Differences in antibiotic sensitivity were not observed within these mixtures containing type 80/81 strains, although some variation occurred between strains from different specimens. The degree of pigmentation did not correlate with sensitivity or resistance to phage 44A.

DISCUSSION

The observed 7 per cent incidence of mixed growth represents a lower limit since primary detection is based on a single property, pigmentation, and is not of sufficient sensitivity to detect mixtures in which one component occurred in proportions lower than 1 per cent. The ratios may also have been altered by the preliminary, but unavoidable, subculture. The method is sensitive enough, however, to detect mixtures not apparent on the original blood agar plates. The proportion of samples with mixed growth agrees closely with that observed by Bynoe and associates[7] but is considerably less than the 12 per cent observed by Wallmark.[8] When mixtures are not recognized as such and are regarded as pure strains, the laboratory findings may be misleading. Bacteriophage typing and antibiotic sensitivity testing of an isolated colony would give a different set of results than similar tests performed on the mixed growth. This may be of particular importance if the two strains differ in virulence and treatment is based on the laboratory findings for the strain of lesser virulence. The possibility of mixed infection should be considered in instances in which response to therapy does not coincide with laboratory findings of antibiotic sensitivity.

The lysogenic state, common in coagulase-positive staphylococci, undoubtedly influences the prevalence and stability of such mixtures, since phage carried by one strain may lyse or lysogenize the other. A superinfecting strain can thus have a transient existence, can replace the original strain, can interact to produce new types, or may coexist as a stable mixture.[9] Such interactions may be a factor in the variability of type 80/81 which has rapidly spread and colonized in hospitals throughout the world.[4, 10] If this type superinfects the nasal passages of a staphylococcus carrier, the interaction and lysogenization may produce the variation which we have observed. Another possibility is that type 80/81 has a higher spontaneous mutation rate than other strains of staphylococci. Variation in sensitivity to antibiotics reported from different localities for this type[4] might be evidence of genetic instability.

SUMMARY

1. Fourteen of 196 routine clinical specimens containing coagulase-positive staphylococci were shown to be mixtures of two strains.

2. The pairs of strains in these mixtures differed in pigmentation, sensitivity to one or more antibiotics, and in bacteriophage typing pattern.

3. Strains of bacteriophage type 80/81 showed a relatively high degree of variation in pigmentation and in bacteriophage typing pattern.

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ROLE OF ADRENAL HORMONES IN LYMPHOCYTE MOBILIZATION IN THE RAT

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IN A previous study we described a method for replacement-transfusion of rats with donor blood that had been rendered leukocyte free.[1] Rapid removal of blood was achieved by this technique, and the rate of leukocyte mobilization from storage areas for replenishment of the circulating blood leukocyte levels could be studied. The remarkable thing about these experiments was the extreme mobility of leukocyte reserves in the rat, so that total replenishment of the circulation occurred within 10 to 30 minutes after cessation of active leukocyte removal. Repeated periods of leukocyte removal failed to exhaust the reserves. Equally striking was the observation that both granulocytes and lymphocytes were brought back into the blood stream in quantities necessary to restore their original levels.

From these observations, we concluded that the rat has large pools of readily available, preformed leukocytes. Because of the rapidity with which these leukocyte reserves entered the blood stream, it seemed clear that they were in or near vessels from which they could enter the general circulation.

In the previous studies, we found no clue to factors that might control the magnitude or mobility of these leukocyte reserves. Many studies have indicated the important role of the adrenal hormones in the quantity of lymphoid tissue, and Dougherty and White[2] have emphasized the lymphopenic effects of adrenal hormones in rodents. Since the leukocytes of the rat are mostly lymphocytes, the present study was undertaken to determine what effects, if any, hypophysectomy, adrenalectomy, or cortisone therapy would have on the readily mobilized reserves of lymphocytes in the rat.

METHODS

Sprague-Dawley or Sherman strain rats were used in all experiments. Heparinized donor rat blood was obtained by cardiac puncture, was centrifuged, and the buffy coat removed by the methods previously described.[2] The reconstituted donor blood regularly had leukocyte counts of less than 500 per mm^3.

The *replacement transfusions* were *performed* using the previously described technique. Donor blood was injected into a femoral vein plastic cannula at a rate equal to the spontaneous outflow from a cannula in the femoral artery. Replacement transfusions of 20 ml., found to represent at least 80 per cent blood volume replacement,[1] were accomplished within 5 minutes or less.

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Total and differential leukocyte counts from blood samples obtained from the arterial cannula were made before, immediately after, and at varying times during the recovery phase.

Replacement transfusions were performed on rats weighing approximately 250 grams. In the experimental groups were: (1) 5 rats of the Sherman strain that had been adrenalectomized 1 to 11 days before study, and had been maintained with the subcutaneous administration of 0.5 mg. of desoxycorticosterone acetate (DOCA) daily and 5 per cent glucose in saline for fluid intake; (2) 5 rats of the Sprague-Dawley strain that were hypophysectomized approximately 2 weeks before use*; and (3) 7 rats treated with 5 mg. cortisone acetate (Merck) subcutaneously for 14 days before study. Although extensive experience with normal animals had been gained in previous studies, appropriate sham-operated or injected rats were used as controls. In daily experiments, both an experimental and a control animal were subjected to replacement transfusion with identical donor blood.

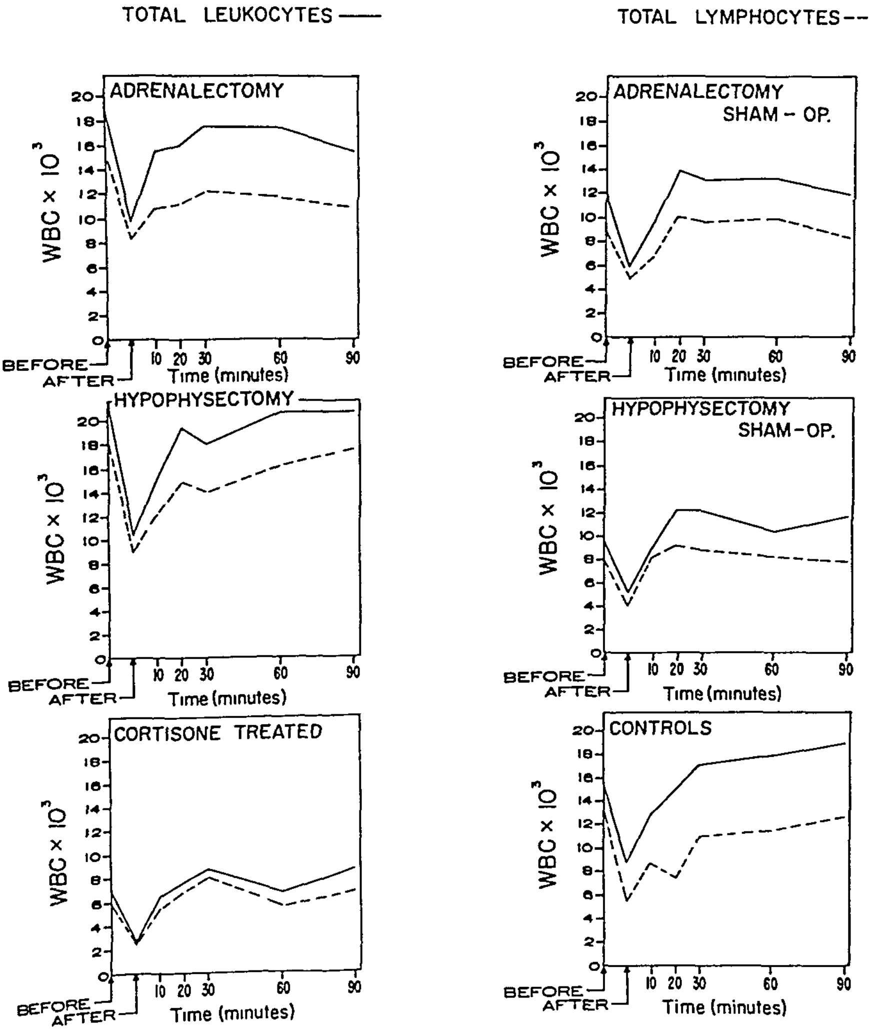

Fig. 1.—Mean changes in leukocytes and lymphocytes during and following leukocyte replacement transfusion.

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*Obtained from the Endocrine Laboratories, Madison, Wis.

RESULTS

As anticipated, the peripheral blood leukocytes (predominantly lymphocytes) were increased in adrenalectomized and hypophysectomized animals as a concomitant of their increased lymphatic tissue. Conversely, the cortisone treated animals had low total leukocyte and lymphocyte levels correlating with the general decrease in lymphoid tissues following adrenal hormone therapy. These differences were marked, with the mean total leukocyte level of the adrenalectomized group 19,100 mm^3, of the hypophysectomized group 21,600 mm^3, and of the cortisone treated groups only 7,200 mm^3. The marked leukopenia and lymphopenia of the cortisone treated rats is statistically significant ($P>0.05$), using the ranking test of White,[3] when compared with either the adrenalectomized, hypophysectomized, or the control saline injected rats.

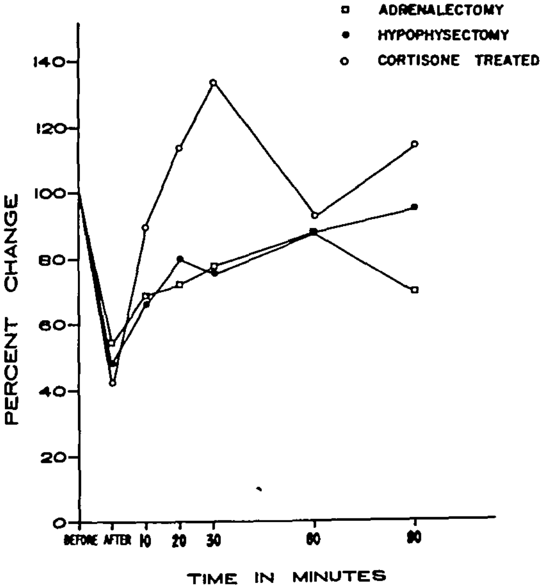

Fig 2—Comparison of the percentage change in lymphocytes of adrenalectomized hypophysectomized and cortisone treated rats

These 3 groups of animals with markedly different lymphatic tissues were subjected to leukocyte replacement transfusion. The results of mean total leukocytes and total lymphocyte changes are shown in Fig 1. In all groups, considerable mobilization of leukocytes (mostly lymphocytes) occurred during the approximately 5 minutes required to complete the transfusion procedure

This acute mobilization of cells was manifested by the fact that the calculated 80 per cent blood volume replacement resulted in only 60 per cent or less decrease in leukocytes. Recovery to approximately initial levels occurred in all instances in 20 to 30 minutes, but no significant increases above initial levels were *noted. Although the cortisone-treated group had the low total* lymphocyte count, the lymphocyte recovery curve, calculated as per cent of initial value, appears even more brisk than in the other groups (Fig. 2). Even the quantitative mobilization during the first 30 minutes (increase of approximately 5,000 lymphocytes per mm.^{3}) is not inconsiderable.

DISCUSSION

Adrenalectomy and hypophysectomy caused the expected increase in peripheral blood lymphocytes in the rat, and cortisone therapy resulted in a marked lymphopenia. When lymphocytes were removed during the course of a total leukocyte replacement transfusion, the cortisone-treated rat exhibited good ability to mobilize lymphocytes back to the initial circulating level. It would seem, then, that cortisone therapy, although altering the total lymphatic tissue, does not significantly change these lymphocytes that we have termed the ''ready reserve'' for blood entry.

Of interest was the fact that this brisk mobilization of lymphocytes in the cortisone-treated rats did not exceed the initial value. The mobilization, then, ceased rather abruptly at the original lymphopenic level, without any tendency to return to normal or even higher leukocyte levels. We have noted this type of ''thermostatic'' control of circulating blood leukocyte levels in other studies. The adrenals play a presumably significant role in setting this level for the circulating lymphocytes in the resting animal and, if so, would logically be responsible for controlling the cessation of rapid mobilization in response to our procedure.

In none of our studies to date have we been able to significantly exhaust the leukocyte reserves of the rat, regardless of modification of the animals by operation or drugs. Since the total magnitude of the lymphocyte stores has not been determined by our method, they might be markedly depleted in the cortisone-treated animals that seem to respond so readily. Gowans[4] certainly has demonstrated that chronic removal of lymphocytes from the circulation by thoracic duct drainage does cause eventual lymphopenia.

The significant aspects of these observations, however, is the fact that depletion of circulating lymphocytes by cortisone does not abolish these readily mobilizable cells. The biologic functions of such ready reserves are not clear, but the fact that they seem to remain, even in the animal depleted in lymphoid tissue, suggests that they are of some importance to the rat.

SUMMARY

1. The effects of increased lymphopoiesis (hypophysectomy and adrenalectomy) and of decreased lymphopoiesis (cortisone therapy) on the response of rats to sudden depletion of blood leukocytes by replacement transfusion was studied.

2 Depletion of leukocytes by this technique was followed by a return to essentially base-line levels within 30 minutes as lymphocytes were rapidly mobilized from body stores

3 Even severely lymphopenic rats (cortisone-treated) exhibited rapid mobilization of lymphocytes, although there was little tendency to exceed the initial blood levels

We would like to thank Miss Sarah E Mair for technical assistance in this study.

The actual leukocyte values obtained in the individual experiments are available from the author on request

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LABORATORY METHODS

PRODUCTION OF MAGNETICALLY CONTROLLED VASCULAR OBSTRUCTION

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IN STUDIES necessitating the occlusion of blood vessels there is a need for a controllable and nontraumatic method of obstruction. Clips or ligatures are frequently used on large vessels, with resulting local trauma. It is possible to obstruct small vessels by the injection of microspheres, which eliminate trauma, but generally obstruct all small vessels.[1] Heat and chemicals have been used to infarct capillary beds, but these are extremely traumatic and may obscure the pathology.

The technique developed here employs a magnetic field applied to the exterior of blood vessels, through which small ferromagnetic particles are passing. The field holds the particles until a sufficient collection completely obstructs the vessel. Early release of the fields eliminates the holding force, and the particles are free to leave the site, while a more prolonged application leads to permanent occlusion of the vessel. Magnetic fields have been used in biologic experiments for the separation of phagocytic cells after they have engulfed ferromagnetic particles,[2] but they have not to our knowledge been used to produce vascular obstructions.

MATERIALS AND METHODS

The method consists of the injection into a suitable blood vessel of a solution of 1 per cent gum acacia in an isotonic saline solution containing a 5 or 10 per cent suspension of magnetic ferric oxide (Fe_3O_4).[†] The estimated dosage of ferric oxide is approximately 20 mg. per kilogram. Observations suggest that dosages over 50 mg. per kilogram may result in a delayed death due to an excessive accumulation of iron in the lung and reticuloendothelial system. The particles of oxide being smaller than the red corpuscles (1 micron) easily pass through the capillaries and, inappropriate dosage, through the lung without obstruction. After the injection, an electromagnet (described below) is applied over, but not in contact with, the exposed vessel or area to be infarcted. When the vessel is of sufficient size, the accumulation of oxide can be easily visualized beneath the magnet. When the magnetic field is removed prior to the development of a permanent obstruction, the accumulation of

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[†] Cat. No. 1-119 Fe_3O_4; Fisher Scientific, Pittsburgh, Pa.

oxide can be seen to break apart and be carried off by the restored flow of blood If the magnetic field is promptly reapplied, the oxide may again be accumulated in the field but usually in a smaller quantity

Two different types of magnets have been employed in this study Type I is designed to produce a high intensity field in a carefully restricted area for obstruction of a selected vessel This uses a ¼ inch soft iron rod folded upon itself with the free ends converging leaving a 4 mm air gap at the tip The magnetic field produced by 5,500 turns of No 28 copper wire wound around the rod is concentrated in the 4 mm gap between the poles Power is provided by a 28 v D C 2 amp power supply The gap formed by the converging filed ends of the ¼ inch rod is just large enough to admit the blood vessel to be obstructed The coronary artery was actually dissected free and drawn between the poles by a Teflon tape secured to the magnet Superficial cerebral vessels may be admitted between the poles by gently pressing the poles again at the brain surface, thereby depressing the tissue adjacent to the vessel

Magnet Type II is designed to produce a widespread magnetic field for obstruction of all vessels of a tissue area, rather than obstruction of a specified blood vessel This has been used to infarct vessels in the spinal cord and brain The original Type II Magnet was a commercial surgical eye magnet, consisting of many turns of copper wire on a straight iron rod The magnetic field thus produced radiates from the tip of the rod A new magnet has been prepared with a longer tip for easier application to less accessible areas An air cooling coil has also been incorporated into the design so that the field may be applied for a sustained period without overheating

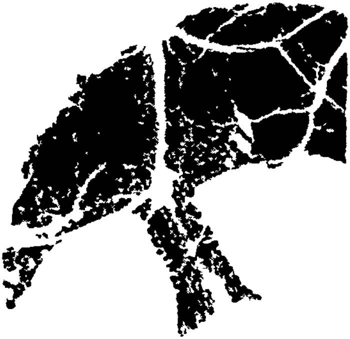

Fig 1 —Arterial obstruction in a rabbit s ear following the discontinuance of the electromagnetic field The small obstruction between the magnet poles represents a portion of the main block being carried peripherally by the returning blood flow (Reproduced from a motion picture frame)

RESULTS

Preliminary studies with this technique were carried out utilizing a large artery in a rabbit's ear (see Fig 1) The cerebral cortical veins (Fig 2) and arteries of 3 large dogs and a rhesus monkey were subjected to temporary and permanent infarctions In these experiments the oxide was injected into either

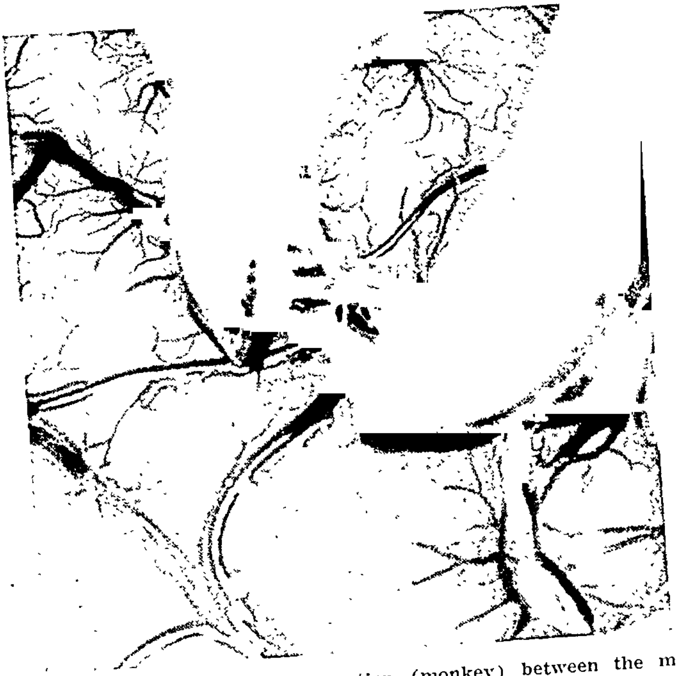

Fig. 2.—A cerebral cortical venous obstruction (monkey) between the magnet poles.

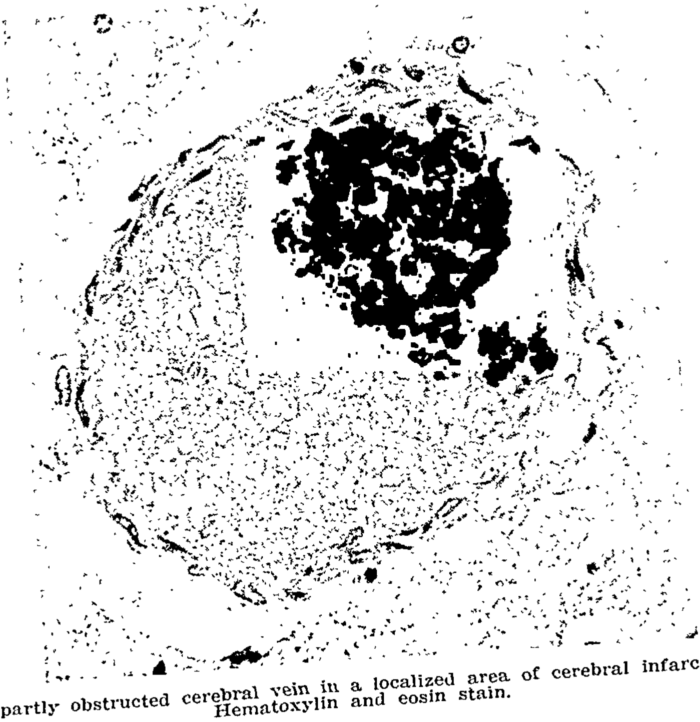

Fig. 3.—A partly obstructed cerebral vein in a localized area of cerebral infarction in a dog. Hematoxylin and eosin stain.

the saphenous vein, pulmonary artery, or carotid artery. The site of injection seems to have no appreciable effect on the time of development or size of the obstruction. When the magnetic field was maintained for 10 minutes or longer the obstruction remained permanent. Limited observations seem to indicate that heparin injections may further prolong this period of reversibility. With Magnet Type II, localized areas of infarction were produced in one cerebral hemisphere of a dog (Figs. 3 and 4).

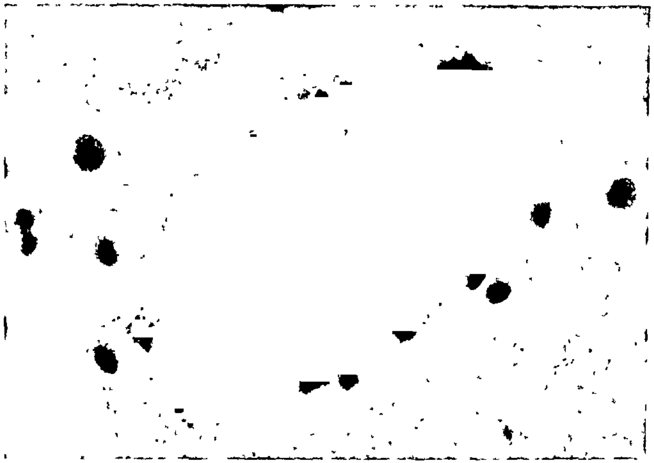

Fig. 4.—A completely obstructed cerebral vein in an area of localized infarction in a dog. Hematoxylin and eosin stain. (Magnification ×640, reduced ⅔.)

Two attempts were made to produce a coronary occlusion in large (30 Kg.) dogs by the injection of 7 ml. of 10 per cent oxide solution into the saphenous vein. The magnetic field was maintained for 15 minutes over the exposed anterior coronary artery. No appreciable accumulation of oxide could be observed, and electrocardiographic recordings showed no change within a 30-minute period of observation. A second injection of 8 ml. was then made and the field maintained for 10 minutes with no demonstrable effect. Electrocardiographic observations over a 1-hour period failed to demonstrate any changes that could be ascribed to the injection. The animal was then sacrificed and histologic studies of the heart also showed no evidence of occlusion. A repetition of this experiment with modification of the magnet shape to permit a better application of the magnetic field was also unsuccessful. It is our opinion, however, that coronary occlusion may be produced by utilizing a stronger magnetic field or in an experimental animal having smaller coronary vessels.

By the application of the magnetic field to the exposed spinal cord (thoracic) of a monkey and a dog it has been possible to produce an area of localized infarction. The occlusions in the superficial vessels of the cord can be easily observed and microscopic studies also demonstrate (Fig. 5) the occlusion of the smaller intraspinal vessels. Further studies on spinal cord infarction are in progress.

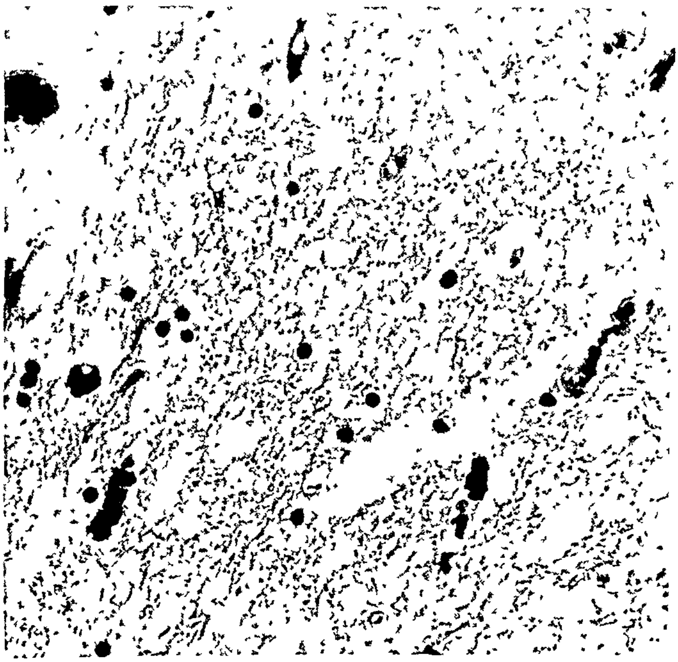

Fig. 5.—Generalized obstruction of all vessels in a localized portion of the midthoracic spinal cord of a dog Hematoxylin and eosin stain

Of the 9 animals used in these experiments (1 rabbit, 6 dogs, and 2 monkeys) 3 dogs were subjected to acute experiments and were sacrificed. Two animals expired (1 dog and 1 monkey) following the procedure, the dog within 30 minutes and the monkey after 8 hours. In both cases the dosage of Fe_3O_4 was 50 mg. per kilogram or over. Histologic studies in each case showed a large accumulation of the oxide in the lungs and reticuloendothelial system. The remaining animals showed no ill effects 3 to 4 weeks after the injection.

SUMMARY

A technique for the transient or permanent occlusion of small arteries, veins, and capillary beds is presented. The method is dependent on the accumulation of magnetic ferric oxide when the vessel or tissue area is subjected to an electromagnetic field. The advantage of the technique may reside in the avoidance of trauma to the vessel and adjacent tissue and in the production of controlled infarctions and ischemia. It is suggested that this technique may be of value in studying electrical and chemical changes in small areas of tissue

subjected to periods of transient infarction It is hoped that the technique can be improved with further development of the electric magnet in regard to the strength and localization of the magnetic field

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We currently use rats weighing 40 to 50 grams; this enables us to detect 0.1 rat unit or less. With these rats it is necessary to inject only small volumes of fluid at a time. We operate within the range 0.01 to 0.1 ml. using a 1 ml. tuberculin syringe driven with a micrometric screw attachment (Fig. 2). The assembly permits the rapid interchange of syringes and is very accurate. One full revolution of the micrometer permits the injection of approximately 0.02 ml. Since all our measurements are relative, we operate in terms of revolutions of the micrometer.

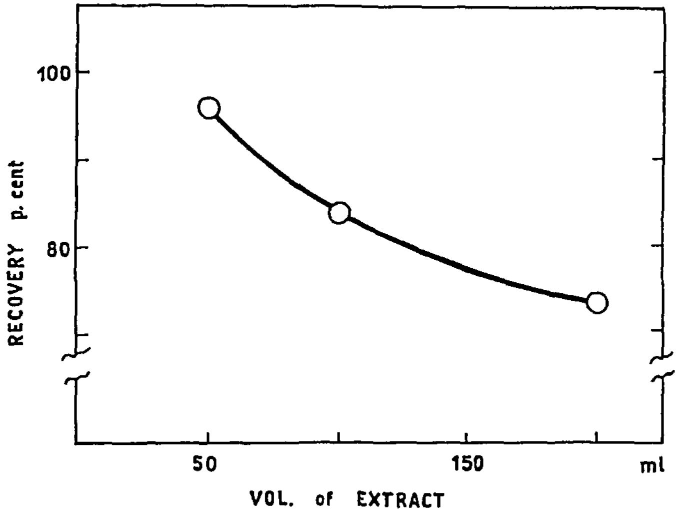

Fig. 1.—Four hundred milliliters of dog blood was processed as described in the experimental procedure up to the stage of passage through the column. The 400 ml. extract obtained was then divided into 4 samples of 50, 50, 100, and 200 ml. One 50 ml. sample was used as a blank and to each of the others 10 units of angiotensin was added. All the samples were then percolated through identical columns of Dowex 50-X2, of 10 cm. per 1 cm. The rest of the procedure was as described. The experimental points represent the recovery of added angiotensin expressed in percentage after deduction of the angiotensin contained in the blank.

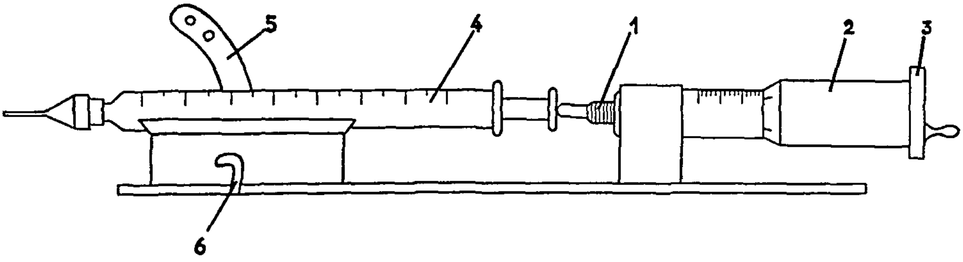

Fig. 2.—Micrometric attachment for the injection of small volumes of fluid to the rat. Key: 1 = micrometric screw of 1 mm. pitch; 2 = barrel graduated in turns and one-fourth turns; 3 = driving head; 4 = 1 ml. tuberculin syringe, 5 = rubber band, 6 = pin to clamp the rubber band and fasten the syringe to the base.

The dead volume that exists in the polyethylene catheter between the blood stream in the jugular vein of the rat and the extreme of the needle of the syringe must be filled with the solution to be assayed before any test injection can be made. Usually this volume does not exceed 0.01 ml.

The routine procedure employed to measure the pressor activity of an unknown solution consists, first, in adjusting the volume injected so as to obtain approximately equal pressor responses in comparison with the standard solution; and, second, in making injections in the following order: 2 or 3 doses of the standard, 2 of the unknown, and the same 2 or 3 doses of the standard. If there is a marked difference between the first and the last set of standards, the whole series is repeated until the 4 or 6 injections of standard give similar responses. The simple graphical procedure used to determine the activity of the unknown in terms of the standard is explained in Fig 3.

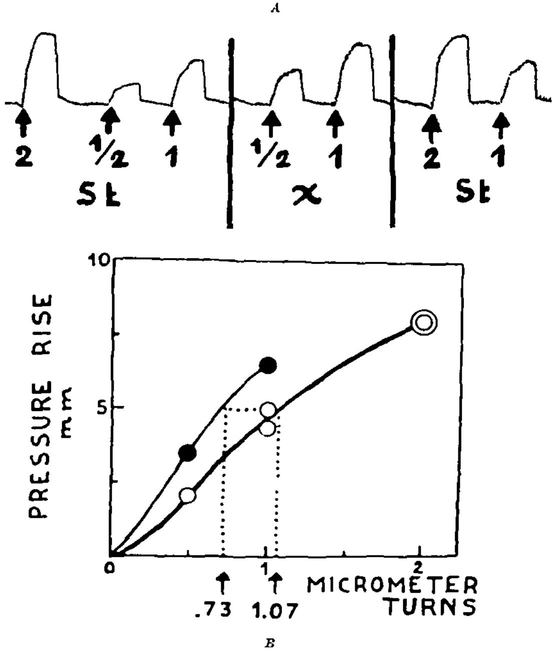

Fig 3.—Routine procedure to estimate the pressor activity of unknown solutions in terms of an arbitrary standard. *A*, Pressor responses to successive injections of standard angiotensin (5 rat units per milliliter) and unknown solution. The numbers indicate the turns of the micrometric screw corresponding to the injection. *B*, Graphic representation of the pressor responses measured in millimeters of mercury, as a function of the amount injected. *Open circles*, standard angiotensin points, *black dots*, unknown angiotensin solution points. It is clear that 0.73 of the unknown is equivalent to 1.07 of the standard. Hence the concentration of the unknown is 7.3 rat units per milliliter.

RESULTS

To test the validity of the procedure, 2 accurately made dilutions of the concentrated mother solution of the standard angiotensin were assayed on 11 and 10 animals, respectively, against the usual standard. The results (see Table I) demonstrate that the method has a maximum error of ±14 to 21 per cent on single measurements. The standard deviation in both series does not exceed 11 per cent.

Recovery Experiments.—All the experiments reported were done by adding 10 rat units of angiotensin to 50 ml. of heparinized dog's blood just prior to its mixing with the ethanol, as explained in the section on Methods. Another 50 ml. of the same blood was processed at the same time and the pressor value obtained was considered a blank of the first sample. Special care was taken to operate with both samples almost simultaneously in the first step to avoid any error due to the observed increase of the blank value that takes place in heparinized blood at room temperature. These data, collected in Table II, show that the method permits a complete recovery of the added angiotensin. The variability observed in single determinations can be explained in terms of the error due to the biologic assay.

TABLE I. REPRODUCIBILITY OF THE ASSAY METHOD

R.U./ML. THEORETICAL	R.U./ML. FOUND (MEAN VALUES)	STANDARD DEVIATION	R.U./ML. EXTREME VALUES FOUND	NO. OF MEASUREMENTS
7.50	7.43	0.64	6.45 8.55	11
3.00	3.24	0.33	2.59 3.63	10

R.U. = rat units.

TABLE II. RECOVERY EXPERIMENTS

NO. OF EXPERIMENT	TOTAL RAT UNITS FOUND	RAT UNITS OF BLANK	RECOVERY (PER CENT)
1	11.4	2.2	92
2	12.0	2.2	98
3	12.7	3.3	94
4	12.5	3.3	92
5	13.6	3.3	103
6	12.4	2.6	98
7	13.5	2.6	109
8	14.2	2.6	116
9	12.3	1.7	106
10	16.2	5.1	111
11	14.4	4.8	96
12	15.9	6.5	96
		Mean recovery	100.9
		Standard deviation	8.1

Specificity of the Method.—Recovery experiments were performed as described before, but other pressor substances were added to the blood. Amounts of adrenaline or noradrenaline equivalent to 25 rat units gave samples devoid of pressor activity. The various alkaline treatments of the procedure must be responsible for their destruction. On the other hand, pepsitensin[14] was completely recoverable, a fact that emphasizes its close similarity to angiotensin. Tyramine and vasopressin* were recoverable only to the extent of 15 per cent of the added amounts. The recovery of serotonin was not attempted since our biologic preparation was constantly insensitive to it. The possibility that tyramine or vasopressin could appear in the final extracts was routinely excluded by testing the resistance of the pressor activity to destruction by trypsin and thioglycollic acid. As is well known, tyramine is unaffected by trypsin while vasopressin and angiotensin are completely destroyed. Thioglycollic acid, on the other hand, has no action on tyramine and angiotensin but selectively destroys vasopressin.[15]

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*Pitressin: Parke, Davis & Company, Detroit, Mich.

The incubating mixture for the trypsin test was as follows:

Extract	0.20 ml.
Phosphate buffer 0.5 M, pH 7.5	0.015 ml.
Merthiolate 4 mg./ml.	0.005 ml.
Crystalline trypsin (Armour) 5 mg./ml.	0.015 ml.

In 10 minutes at 38° C. this proportion of trypsin was sufficient to destroy at least 3 rat units of angiotensin. At the end of the incubation period, the mixture was injected into the rat without further treatment, since it was found that a similar mixture with saline replacing the extract had no effect on the blood pressure of the rat.

For the thioglycollic acid test, the incubating mixture was identical to the previous one but the trypsin solution was replaced by 0.020 ml. of a 0.1 M solution of thioglycollic acid in 0.1 M sodium bicarbonate, final pH 7.5. The incubation time was 30 minutes at room temperature. Under the described conditions, an absolute amount of vasopressin equivalent to 2 rat units of angiotensin is completely inactivated.

DISCUSSION

The problems encountered in the quantitative determination of angiotensin in blood are many. One is that the withdrawal of blood may cause a decrease in blood pressure, thus inducing the secretion of renin by the kidney[11] with the subsequent formation of angiotensin. By reducing the size of the blood samples to 50 ml. this cause of error has been completely eliminated.

The small blood sample, which may eventually be increased to 75 or even 100 ml. according to the size of the animal, creates another problem. The amount of circulating angiotensin which one should expect in hypertensive animals must be very small. Skeggs and associates[9] found from 0.1 to 1.6 Goldblatt units per liter of blood in dogs with acute malignant hypertension and from 0 to 0.05 unit per liter in normal dogs. The fact that the recovery in our method is 100 per cent, while in the method of Skeggs and co-workers[9] it is only about 50 per cent, already increases the sensitivity of our method. The final sample in our method is usually 0.5 ml.; however, it may be reduced to 0.1 ml. and injected totally in a rat weighing 50 grams, thus increasing the sensitivity of the method four- or fivefold in cases where the concentration of angiotensin is expected to be very low. In a rat of this size we can easily detect 0.00054 Goldblatt unit (which would mean for a 50 ml. blood sample 0.01 Goldblatt unit per liter of blood).

The advantage of using a 50 gram rat is that, assuming a concentration of angiotensin of more than 0.03 unit per liter of blood, a fairly good quantitative assay and identification test can be made.

Preliminary experiments have allowed us to determine quantitatively the circulating angiotensin in: (1) nephrectomized animals subjected to a continuous infusion of angiotensin, (2) normal animals injected with renin, and (3) normal animals subjected to variable periods of deep hypotension.

Experiments are now in progress in which the estimation of angiotensin in blood is made under different physiologic and pathologic conditions.

SUMMARY

A method has been described for the isolation, assay, and identification of angiotensin from the blood. A 50 ml. arterial blood sample is collected into ethanol and the angiotensin removed from the filtrate by the resin Dowex 50-X2. On elution with alkali a solution is obtained which can be further concentrated and assayed for its pressor action by intravenous injection into small rats. The advantages of the method are the small blood sample used, its high recovery rate, the possibility of making identification tests, and its relatively high sensitivity.

We wish to express our appreciation to Miss Elsa Polillo for skillful technical assistance in the biologic assays.

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ADDENDUM

On more extensive use of the method described since our submission of the original manuscript, we have found and solved two experimental difficulties that can lead to low recoveries of angiotensin.

The first problem arose when we used a different batch of Hyflo Supercel which showed a high capacity to adsorb angiotensin. Accordingly, we decided to substitute the second and third filtrations of the method for centrifuging at 12,000 g for 10 minutes in each case. The first filtration was not critical even with the adsorbent batch of Hyflo Supercel.

The second problem arose after the resin had been used repeatedly. Under these circumstances, not even the alkaline treatment in the steam bath could fully restore its original capacity. It is advisable, then, to use fresh resin or alternatively equilibrate the old with citrate buffer 0.2 N, pH 3.1[13] and percolate the sample adjusted to the same pH.

A NEW PRECIPITATION METHOD FOR ESTIMATING SERUM BETA LIPOPROTEINS

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CHARLESTON, S. C.

THE correlation between elevated serum cholesterol, serum beta lipoproteins, and the incidence of thrombotic complication of atherosclerosis has been accepted by most authorities. A simplified method for estimating serum cholesterol and serum beta lipoprotein levels is presented here.

Several years ago it was observed by one of us (E. B.) that a small amount of serum added to a dilute solution of buffered K-agar caused a stable colloidal precipitate. There appeared to be a heavier precipitate when serum of a known hypercholesterolemic patient was added than when the serum of a normocholesterolemic was added. During the course of more than 5,000 measurements, a procedure was developed that makes possible a screening test for hypercholesterolemic and hyperlipemic patients. This procedure correlates well with the Sf 0-16 (beta-1 lipoprotein) component of serum and satisfies the need for a simple, inexpensive (less than 1 cent per test for materials) clinical test to estimate serum cholesterol. It can be used effectively for large scale epidemiologic studies. Somewhat similar, but technically more difficult, methods have been developed by Bernfeld and Nisselbaum,[1] Bernfeld and others,[2, 3] Scanu and associates,[4] and Antoniades and co-workers.[5]

Heretofore, the technical difficulties in separating and estimating serum beta lipoproteins have made this determination impractical for any but highly skilled personnel in elaborately equipped laboratories. Cohn alcohol fractionation, preparative ultracentrifuge differential density flotation with chemical analysis of fractions, analytic ultracentrifugation, and electrophoretic separation are the accepted methods for estimating beta lipoprotein levels. Therefore, this method could fulfill a great need.

MATERIALS AND METHODS

Reagents.—$NaH_2PO_4 \cdot H_2O$ (A.C.S. Reagent grade).
$Na_2HPO_4 \cdot 7\ H_2O$ (A.C.S. Reagent grade).
K agar, code No. 400 (Baltimore Biological Laboratory, Inc., Baltimore, Md.).

From the Lipid Metabolism Laboratory, Department of Medicine, Medical College of South Carolina, Charleston, S. C.

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[*] Work done during tenure as an Established Investigator of the American Heart Association.

[**] Work done during tenure as a Postgraduate Research Fellow for G. D. Searle & Company.

K Agar is an extract of carrageenin with a sulfate ash content of 39.1 per cent. Carrageenin,[6] from *Chondrus crispus*, is made up of 1,3′ linked D galactopyranose units sulfated on carbon 4 with a sulfate ash content of 19 to 23 per cent. The sulfate is bound as a half ester with the polysaccharide while the remaining portion of the acid is in salt formation with metallic ions, principally sodium, potassium, and calcium. This is significantly different from other agars.

Control Buffer Solution.—Mix equal volumes of 0.0667 M $NaH_2PO_4 \cdot H_2O$ (9.207 Gm. per liter) and 0.0667 M $Na_2HPO_4 \cdot 7H_2O$ (17.873 Gm. per liter).

K Agar Buffer Solution.—Mix equal volumes of 0.0667 M $NaH_2PO_4 \cdot H_2O$ and 0.0667 M $Na_2HPO_4 \cdot 7H_2O$. In this solution dissolve enough K agar to make the concentration of K agar equal to 0.50 Gm. per liter. This solution should be refrigerated while in storage. Freshly boiled, carbon dioxide free distilled water should be used to prepare the solutions.

Equipment.—A colorimeter calibrated to read optical density at 540 mμ, cuvettes with an outside diameter of ½ inch, 0.2 and 3.0 ml. pipettes, a pharmaceutical balance for weighing chemicals, and 2 volumetric flasks for making up solutions are the total equipment necessary.

Procedure.—Into the bottom of each of 2 cuvettes, pipette 0.20 ml. of fresh serum. To the first add 3.0 ml. of control solution. To the second add 3.0 ml. of K agar solution. Mix immediately and read the optical density of each solution at 540 mμ in a colorimeter which has been set with a blank containing control solution without serum. The colloidal solution formed is stable for hours with normal sera, but this is not necessarily true with abnormal sera, so readings should be made within 30 minutes. The optical density reading of the control tube gives the inherent turbidity of the serum which is related to the triglyceride content of the serum. The optical density of the K agar tube is due to the precipitated K agar lipoprotein complex plus the inherent turbidity of the serum. Subtracting the optical density of the control tube from the optical density of the K agar tube gives the optical density difference due to the precipitate and is called hereafter K agar Δ.

All cholesterol measurements were made using the acid $FeCl_3$ method of Zlatkis and associates[7] with the color reagent modification of Rosenthal and co-workers.[8] Ultracentrifugal lipoprotein separations were made using this laboratory's modification[9] of the method of Bragdon and associates.[10]

RESULTS

During the period of study, several factors were found to influence the nature and quantity of precipitate formed, namely, (1) the quantity of serum, (2) the concentration of K agar, (3) the pH of the solution, (4) the age of the serum, (5) the temperature to which the stored serum had been subjected, and (6) the character of the metabolic defect, i.e., hypercholesterolemia, hyperlipemia, or diseases which secondarily cause these conditions. Each factor will be discussed separately.

1. There is a simple linear relationship between the quantity of serum used and the optical density of the colloidal solution formed in the range of 0.01 to 0.60 ml. of serum per 3 ml. of reagent.

2. Using a constant quantity of serum, the concentration of the K agar was varied. The K agar Δ value was determined for each K agar concentration and the results are shown in Fig. 1.

At low K agar concentration, where the ratio of beta lipoprotein to K agar is relatively high, a high turbidity is found. At high K agar concentrations, where the ratio of beta lipoprotein to K agar is relatively low, high turbidity

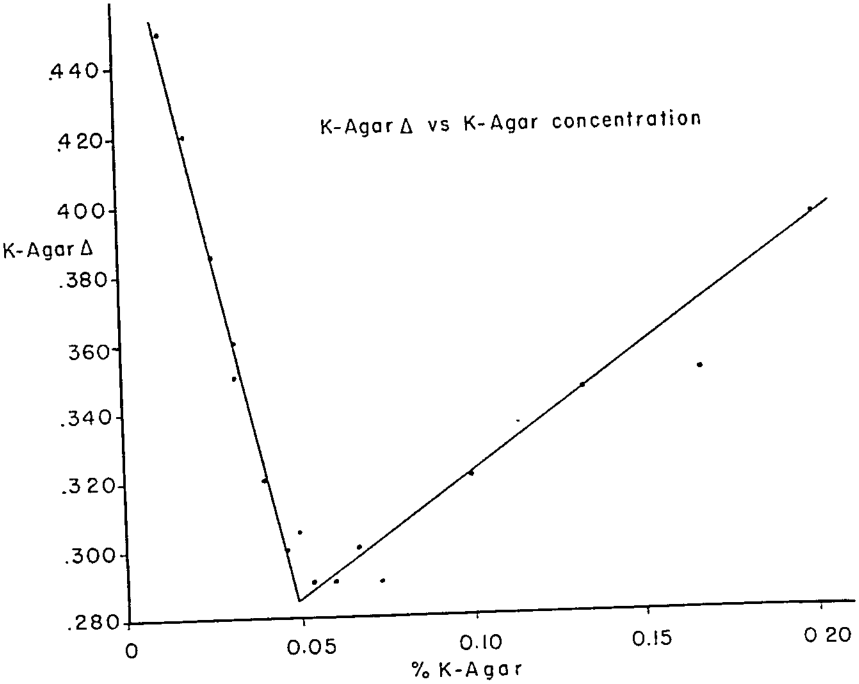

Fig. 1.—K-Agar Δ versus K-agar concentration. See text.

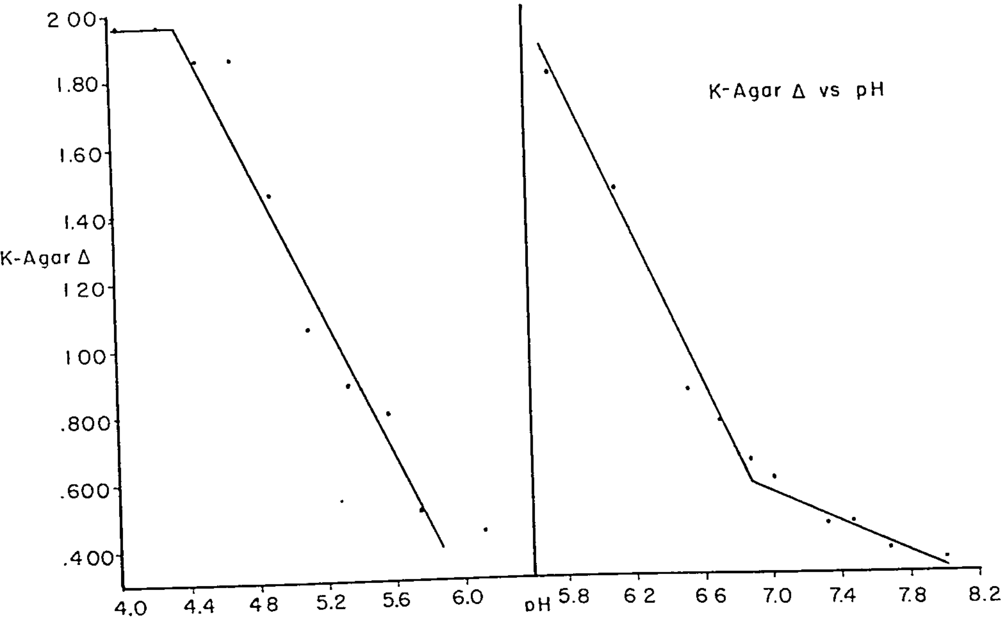

Fig. 2.

values are again found. The curve goes through a minimum at about 0.05 per cent K agar. This 0.05 per cent concentration was selected for the procedure because effects of technical errors in measurements are minimized.

3. The pH of the precipitation medium is important. Fig. 2 is a typical curve showing the relationship of pH to K agar Δ values. Many such curves have been determined and each has a characteristic inflection at a pH in the 6.6 to 7.0 range and a second one near pH of 4.6. The K agar Δ values at the inflection points depend on the concentration of serum beta lipoprotein. The pH chosen for the determination was 6.8 for a number of reasons. At pH values much lower than 6.8 other serum components begin to precipitate. As the pH is raised above 6.8, the amount of precipitate becomes progressively less and the sensitivity of the test is reduced. The advantage of using the 1:1 ratio of salts to make a buffer is obvious in that this ratio gives a maximum stability to the buffering action. When serum is added to the buffer solution there is negligible change in the pH. At pH 6.8 the precipitate is primarily a K agar beta lipoprotein complex. This precipitate, when separated by centrifugation, is easily soluble in dilute ammonia and shows practically no denaturation. This redissolved precipitate migrates and stains as a beta lipoprotein upon paper strip electrophoresis. If the pH of the K agar solution is lowered to 4.0, precipitation of all the cholesterol-bearing lipoprotein components of serum is complete. Tests are incomplete at this stage as to the exact nature of the precipitate at this lower pH, and as to the serum proteins which remain unprecipitated.

4. Lipoprotein lipase activity and other serum lipases cause changes to continue to occur in serum in vitro, therefore, age and temperature of storage will affect K agar values. Shown in Fig. 3 are data on two sera: one hyperlipemic and the other hypercholesterolemic. These two terms are here defined as follows: a serum with an elevated Sf 0-16 low density ($> 1.006 < 1.063$ Gm. per milliliter) beta lipoprotein component, called the β_1 fraction for convenience and simplicity, is defined as a hypercholesterolemic serum. Serum with an elevated Sf 17 and above component, very low density beta lipoproteins, called β_2 for convenience and brevity, is defined as a hyperlipemic serum in which the bulk of serum cholesterol is contained in the very low density (< 1.006 Gm. per milliliter) lipoprotein class and has high triglyceride values. This definition is similar to that of Havel and associates.[11] A hyperlipemic serum, even refrigerated, gives about half as much precipitate at the end of 2 days as it does when fresh. A hypercholesterolemic serum remains fairly stable if refrigerated, but deteriorates at room temperature.

Efforts to stabilize serum were made. Gordon and co-workers[12] demonstrated that unesterified fatty acids will bind tightly to albumin. The use of human albumin is shown in Fig. 3. While some stabilization occurred, albumin was generally ineffective. Also tested with negative results were protamine and high ionic strength, which are known to inhibit clearing (Brown and associates[13]).

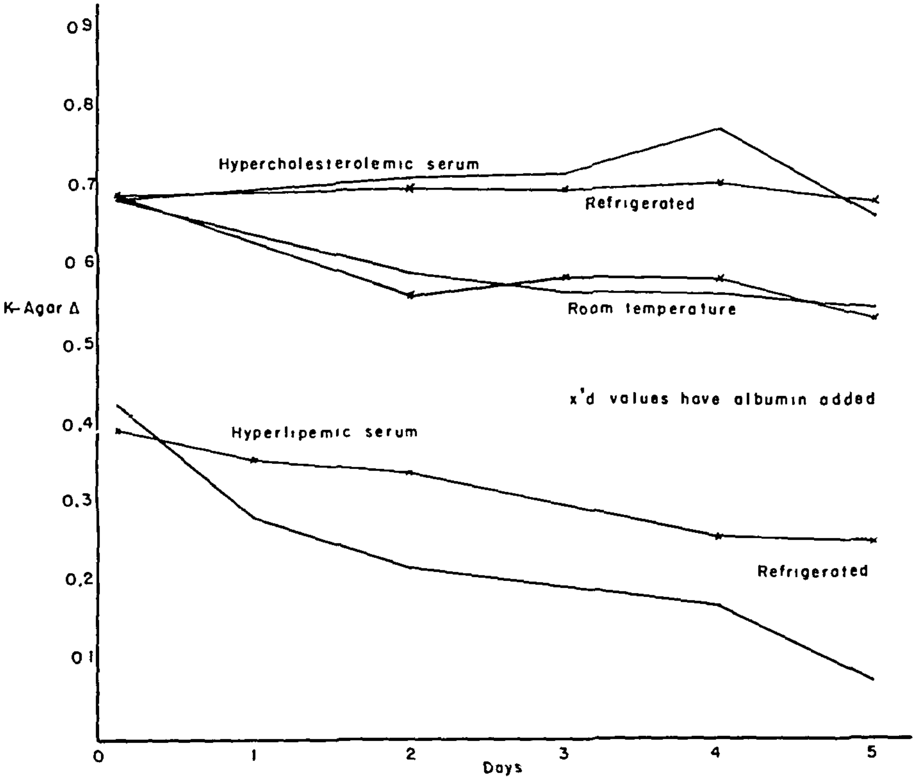

Fig. 3.—K-Agar Δ versus time at room and refrigerator temperatures with and without albumin added.

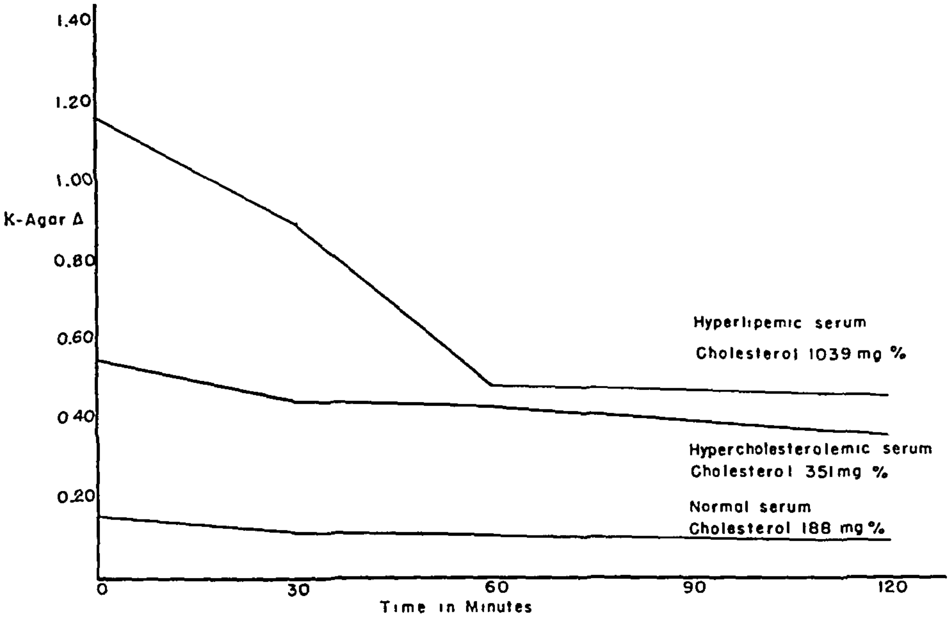

Fig. 4.—K-Agar Δ versus time at 37° C. See text.

5 The variation in values attributable to incubating serum at 37° C before testing is shown in Fig 4 It is interesting to note again that the hyperlipemic serum is more sensitive to heat than hypercholesterolemic or normal serum Also, it is significant that serum cholesterol values do not change measurably in this time period From this discussion, it is apparent that fresh serum must be used for best results using the K agar test

Insufficient numbers of hyperlipemic patients and the rapidity of change of the K agar values with time in such sera prevent the collection of data on the precipitation with K agar of the nonturbid, very low density, β_2 lipoproteins of the Sf 17-400 class

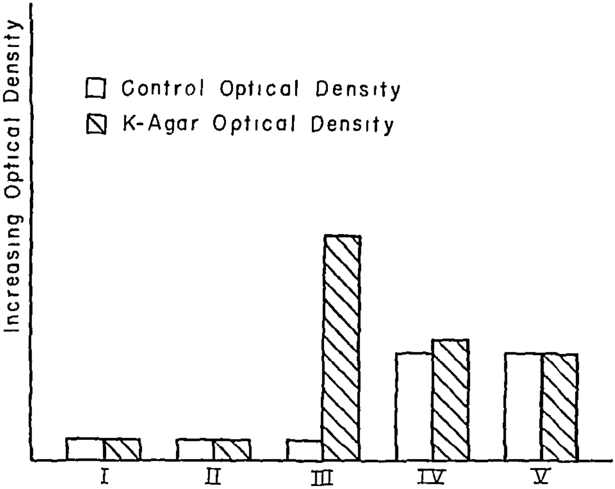

Fig 5—Schematic representation of K agar and control measurement for various lipoproteins Key I = lipoprotein free serum density >1 21 II = high density alpha lipoproteins density <1 21 and >1 063 III = Sf 0 16 beta 1 lipoproteins density <1 063 >1 006 IV = Sf >17 beta 2 lipoproteins density <1 006 V = fresh human chyle

6 In order to characterize the K agar lipoprotein precipitate, serum was fractionated in a Spinco Model L ultracentrifuge giving (1) a lipoprotein free component (the infranatant of 1 21 density cut), (2) a high density alpha lipoprotein component (the infranatant of a 1 063 density cut), (3) a Sf 0 16 component, β_1 (the supernatant of a 1 063 density cut), and (4) a Sf 17 and above component, β_2 (the supernatant of a 1 006 density cut) The schematic results of testing each cut using the K agar test is shown in Fig 5 Also tested was fresh human chyle

For each component a control value and a K agar value is shown If the difference is taken between these values in each case, K agar Δ values can be read. Lipoprotein-free serum and alpha lipoproteins do not cause a K-agar precipitate. The beta-1 component does give a heavy precipitate. The high optical density reading for the beta-2 fraction is due primarily to inherent turbidity as shown by the low K-agar Δ value. Human chyle does not give a detectable K-agar Δ value.

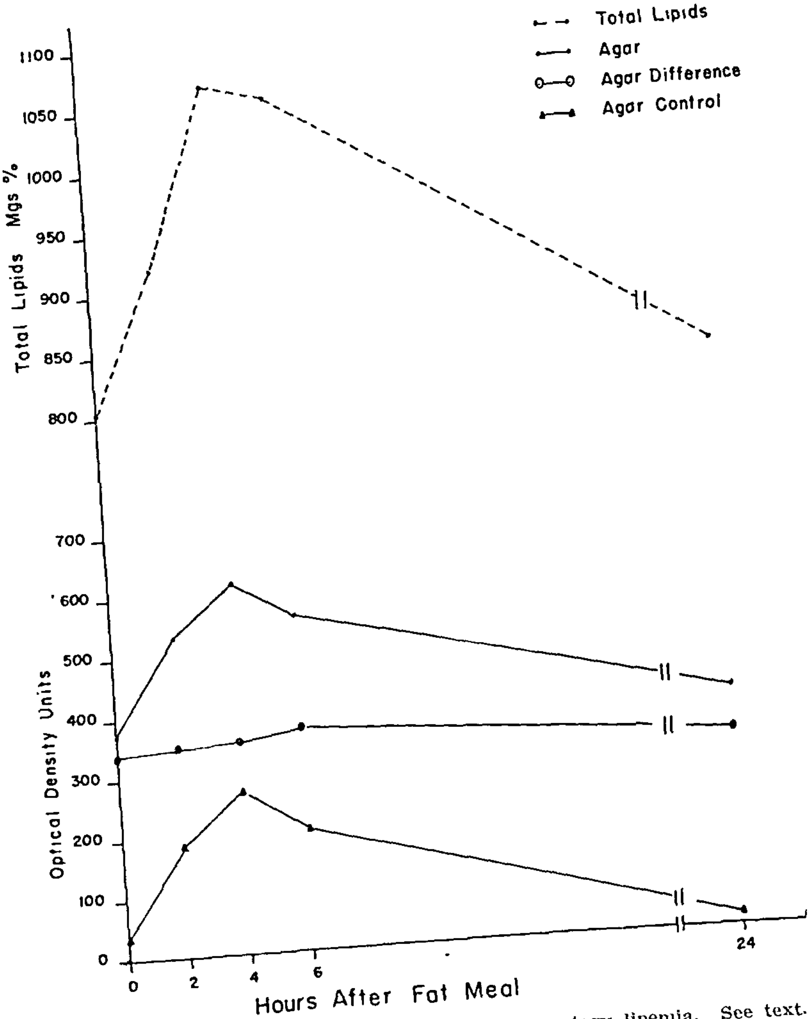

Fig. 6.—Relationship of agar test to alimentary lipemia. See text.

The effect of alimentary lipemia does not destroy the effectiveness of the test. A fat tolerance test was conducted on 24 medical students given orally 1.5 Gm. of fat per kilogram of body weight as a cream milk shake. The composite average of results is shown in Fig. 6.

The broken line shows the serum total lipid values which increase as the fat is absorbed. It is seen that although control and K-agar values increase, K-agar Δ values do not. Although alimentary lipemia does not affect the use of the K agar test on normal subjects and hypercholesterolemic patients, it does make more difficult the diagnosis of hyperlipemia It is recommended, therefore, that the test be made after a high carbohydrate, low fat breakfast, so that endogenous mobilization of body fat for energy does not occur Normal and hypercholesterolemic sera will be clear, and a hyperlipemic serum will be cloudy, ranging from only slightly turbid to very milky

Fig 7 is a scattergraph of 163 beta 1 cholesterol values which were plotted against K agar Δ values Similar scatter was noted when serum cholesterol or total beta cholesterol values were plotted against the K agar Δ

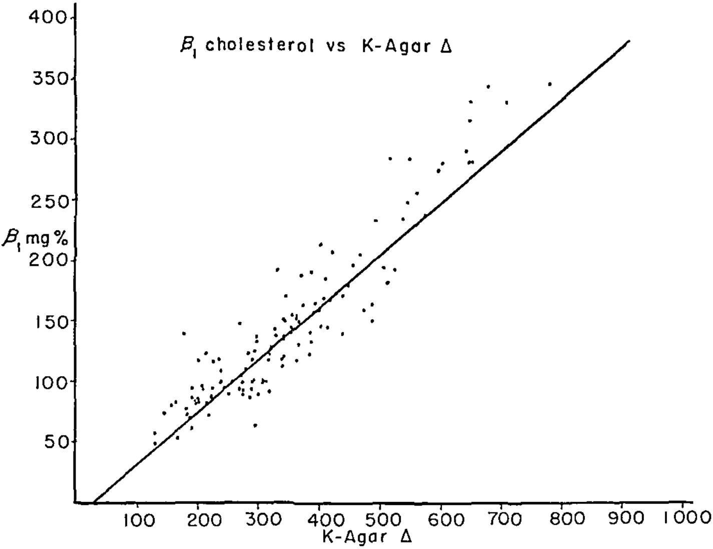

Fig 7—Beta 1 cholesterol versus K agar Δ See text

The calculated regression line is shown In no case was the blood drawn more than 24 hours before the test was made The tests include sera of normal *and some abnormal subjects having either atherosclerosis, biliary cirrhosis,* diabetes, or treated or untreated hypercholesterolemia and hyperlipemic patients

Fig 8 shows the regression lines for total cholesterol (T C), total beta cholesterol (β_1 plus β_2), and beta-1 cholesterol values as plotted against K agar Δ

The statistical data are given in Table I *

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*We are indebted to Dr James A. Gavan Assistant Professor of Anatomy for his help in the statistical studies

TABLE I

REGRESSION EQUATION	REGRESSION COEFFICIENT	STANDARD DEVIATION FROM REGRESSION
T.C. $= 493$ K-agar $\Delta + 70$	$r = 0.934$	27.3
$\beta_1 + \beta_2 = 489$ K-agar $\Delta - 7$	$r = 0.932$	27.2
$\beta_1 = 439$ K-agar $\Delta - 13$	$r = 0.930$	25.0

DISCUSSION

After surveying sera of 1,500 individuals (factory workers, farmers, newborn infants, well-baby clinic patients, student nurses, medical students, hypercholesterolemic patients, and hyperlipemic patients), the upper limit of norms for the following measurements were established: K-agar Δ, 0.350; control, 0.025; serum cholesterol,[7, 8] 240 mg. per cent; total lipids,[14] 900 mg. per cent; alpha cholesterol, 90 mg. per cent; β_1-cholesterol, 140 mg. per cent; β_2-cholesterol, 30 mg. per cent.[9]

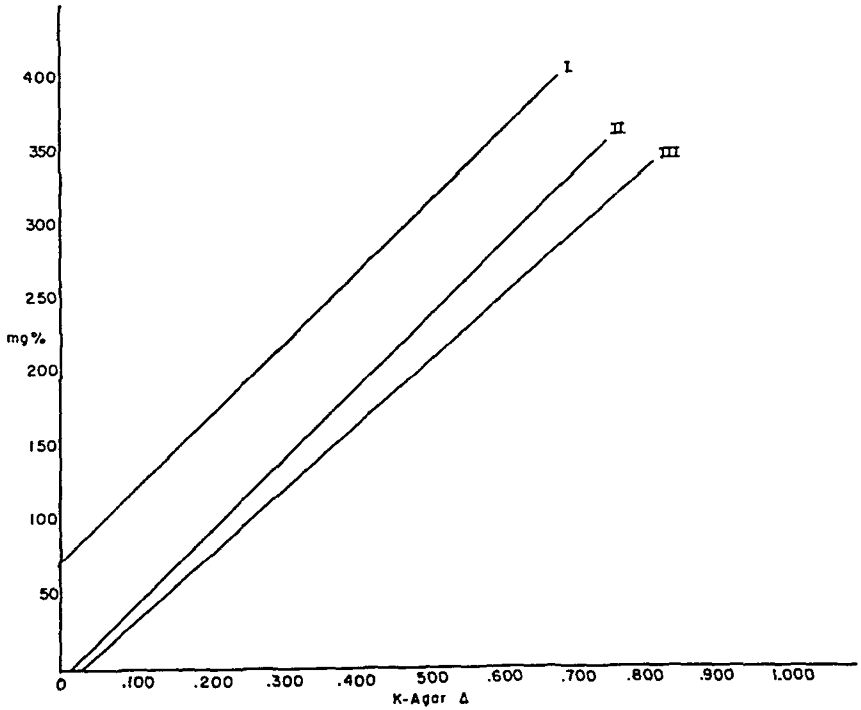

Fig. 8.—Regression lines and cholesterol values versus K-agar Δ. Key: I = regression line of total serum cholesterol values in milligrams per cent plotted against K-agar Δ; II = regression line for the total beta cholesterol values; III = regression line for the beta-1 cholesterol values.

The interpretation of results of the K-agar test is made with the aid of the regression lines in Fig. 8. A clear serum is indicative either of a normocholesterolemic serum or of a hypercholesterolemic serum. A clear serum will give a control value in the normal range. Using the K-agar Δ value and the regression lines of Fig. 8, total cholesterol, beta-1 plus beta-2 cholesterol, and beta 1 cholesterol values may be obtained From these values, by difference, alpha and beta 2 values may be obtained as alpha plus beta 1 plus beta 2 gives total cholesterol

An essential hyperlipemic serum will have a cloudy appearance with a characteristically elevated control value The Kagar Δ value will not correlate well with the regression lines of Fig 8 and the value is generally lower than expected The cloudy appearance of the serum after a high carbohydrate, low fat breakfast is characteristic of a hyperlipemic serum

In this laboratory a high percentage of sera from patients with lipid metabolic disease is seen At least 97 per cent of these patients have hypercholesterolemia, most of the rest have idiopathic hyperlipemia It should be easy for a physician to use this test effectively and with confidence, not only to detect and characterize patients with elevated serum cholesterol, but to follow the results of his treatment

SUMMARY

A new precipitation test for beta lipoproteins (< 1 063 Gm per milliliter) has been described using buffered Kagar Norms have been noted and an interpretation of results has been given Factors influencing the results are described Correlation of this test with serum total cholesterol and serum low density beta lipoprotein levels are made Advantages of such an inexpensive, rapid, simple, and accurate test are described

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A METHOD FOR PREPARING INTACT ANIMALS FOR CARCASS ANALYSES

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BETHESDA, MD.

CARCASS analysis by sampling from a homogenate of the whole animal has met with little success. The primary difficulty has been the disintegration of the skin.

Chanutin[1] ground the carcass in a meat grinder and took aliquot portions for moisture, nitrogen, fat, and ash analyses. No indication was given as to the agreement between duplicates. However, a statement was made that nitrogen analyses showed a marked variation.

Kochakian and Stettner[2] dried the eviscerated animal to constant weight in an electric oven. The dried residue was dissolved in strong alkali and alcohol. The bones were separated and dissolved in acid. The mixture was made to volume and aliquots of the dissolved carcass were taken for nitrogen and fat analyses. Although this method has proved useful for many purposes, it is cumbersome for large animals. Furthermore, the fat is hydrolyzed by the alkaline hydrolysis making it impossible to do any studies on the original lipids, and the ash cannot be determined accurately.

Another method, similar to the above, involved cutting the carcass into small pieces and digesting the material in strong hydrochloric acid at room temperature.[3] When the bones softened, they were put in large beakers and heated on a water bath for several days. "By appropriate manipulation of the volumes and sampling, a solution was finally obtained representing whole animals and suitable aliquots of this were taken for analysis." This method is rather cumbersome and not readily adaptable to animals having a large amount of body fat.

Other workers[4-6] dried the whole animal to constant weight and extracted the fat from the dried residue. The dried defatted residue was ground, and aliquots were taken for nitrogen and ash analyses. This procedure is time consuming, and no duplicate analyses can be obtained for moisture or fat.

The method to be described obviates most of these difficulties since a homogenate of the entire carcass is prepared. After autoclaving the carcass, a slurry can be prepared in a Waring blendor, and a stable suspension results from passing the slurry through a colloid mill. The homogenate can be used for replicate analyses for the 4 major body components (moisture, protein, fat, and ash) as well as for those specific compounds which are not affected by the autoclaving.

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MATERIALS AND METHODS

The special apparatus required for this procedure is an Eppenbach colloid mill* The Waring blendor containers were of 3 sizes 250 ml, 1,000 ml, and 1 gallon, the first two fit the regular size blendor, the largest one fits the four speed model and is needed only for animals weighing over 700 grams

Preparation of Carcass for Analysis—The animal was killed by anesthetizing with chloroform, then weighed to the nearest tenth of a gram The abdomen was opened and the gastrointestinal (GI) tract was removed and freed of its contents which were weighed† The operation was performed in a frame whose three sides and top were wet towels This was done to minimize the evaporation of moisture from the exposed tissues Care was taken to minimize the loss of blood The GI tract was replaced in the animal and the latter was then weighed The actual weight of the animal without GI contents was essentially the same as the weight of the anesthetized animal minus the weight of the GI contents, consequently, the latter was used as the "corrected weight" in our calculations of total body composition

The animal was placed in a wide mouth Mason jar whose volume in milliliters was at least twice the weight of the animal in grams The jar was covered and autoclaved for 15 minutes under 15 pounds pressure (120° C) The animal was then placed in a Waring blendor of appropriate size Water was added to facilitate homogenization In our experience, stable homogenates are secured when the volume of water added approximates, for animals weighing up to 500 grams, the corrected body weight or 0 7 times this volume for animals weighing over 500 grams The exact volume of water does not have to be measured since the weight of the final preparation is used in calculating a factor which corrects for the added water Approximately one half the water was gradually added during the 15 to 20 minutes of blending, the remainder was used to rinse out the blendor and colloid mill

The homogenate was quantitatively transferred to the colloid mill The blendor was washed with small quantities of water and the washings added to the mill Before starting the mill the micrometer adjustment device which controls particle size was set for a reading of 40 The water circulating through the homogenizing block was turned on, the colloid mill was started and allowed to operate for about 3 minutes‡ during which the contents of the mill were stirred to make sure that no particles adhered to the walls of the hopper The micrometer adjustment device was slowly turned in the direction of "close" (using 10 unit intervals every 5 to 8 minutes) until a reading of "zero" was reached The mill was allowed to run at zero for another 5 minutes The colloid mill operation required about 30 to 40 minutes

The homogenate was then quantitatively transferred to a previously weighed Mason jar The colloid mill and recycling pipe (if that had been used) were washed several times with small quantities of water and the washings added to the original homogenate The jar was sealed and the contents mixed by shaking The homogenate was allowed to cool to room temperature, and the total weight was obtained Storage at 4° C followed

Description of Homogenate—Five sieves of the following mesh screen sizes, 10, 14, 18, 25 and 35, were stacked in that order with the smallest mesh size at the bottom Five hundred and thirty four grams of homogenate equivalent to 315 Gm of wet carcass were added to the top sieve The sieves were placed under a faucet and a slow stream of cold water was directed on the homogenate to hasten screening The material remaining on the screens was dried at room temperature overnight and weighed the following day All of the homogenate passed through the 10, 14, and 18 mesh screens Only 0 8 Gm or

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*Manufactured by the Gifford Wood Company 420 Lexington Avenue New York, N Y

†The GI contents were removed since these are not really a part of the animal If it is desirable to analyze the intact animal that can also be done

‡When the weight of the animal was less than 100 Gm a piece of plastic was inserted over the outlet to the recycling pipe and the faucet opened The homogenate coming out of the faucet was collected in the original Mason jar and poured back into the mill Recycling in the case of small animals had to be done manually since the volume of homogenate was not enough to fill the recycling pipe

0.3 per cent of the total remained on the 25 mesh screen and 0.5 Gm. or 0.2 per cent on the 35 mesh screen. The material remaining on these two screens was mainly bone and hair, some of which would have passed through had it not been for the congealed fat in which the bone and hair were enmeshed. The homogenate showed no apparent separation of fat or water even after standing for several days at 4° C.

Analytical Methods.—

Mixing homogenate: Whenever a sample was removed from the homogenate, certain precautions had to be observed. The cold homogenate is in a semicongealed state. In order to hasten heating to room temperature and facilitate mixing, the homogenate was mixed in a Waring blendor. Aliquots were not taken directly from the blendor because consistent, but small, variations between top and bottom aliquots were observed. (Part of the inconsistency in composition might arise from the fact that the slurry practically filled the blendor.) To obviate this difficulty, the homogenate was transferred to the original container which was vigorously shaken just prior to the removal of each aliquot.

A variety of analytic methods may be used on the carcass homogenate. The following are presented primarily to illustrate how the analytic procedures were applied in our laboratory.

Moisture determination: Aliquots equivalent to about 10 Gm. of wet carcass* were placed in aluminum dishes, covered with aluminum foil, and weighed. The aliquots were dried in a vacuum oven at 70° C. to constant weight. This required about 45 hours. The weight of the dried material was obtained and the loss in weight was assumed to be moisture.

Total fat determination: The dried residue from the moisture determination was extracted in a Soxhlet apparatus for 24 hours with petroleum ether (boiling point 35° to 60° C.). The bulk of the solvent was recovered in the upper portion of the apparatus after removing the extraction thimble. The fat in the flask was dried to constant weight in a vacuum oven which was usually accomplished by overnight drying.

Protein determination: One to two grams of homogenate was pipetted (using a large bore pipette) into a 30 ml. Kjeldahl flask and weighed. The nitrogen was determined by a modification of Campbell and Hanna's[7] macromethod. After digestion, the contents of the flask were diluted to a previously calibrated 50 ml. mark and aliquots were taken for distillation.

The small aliquots used for the nitrogen determination were necessitated by the large amounts of fat in the obese rats (those weighing more than 700 grams). If the samples from the obese rats were much larger than the above, excessive foaming occurred which resulted in the loss of part of the sample unless special precautions were taken.

Ash determination: The defatted sample from the fat determination may be ashed or a new aliquot used. In the latter case, 10 to 15 Gm. of homogenate† was weighed in a porcelain crucible (5 cm. diameter). The samples were dried in a vacuum oven overnight. If the sample was from an obese rat, the large amount of fat produced excessive foaming in the muffle furnace with a consequent loss. To avoid this difficulty, the crucibles were heated on a tripod to a dull-red color and the contents ignited with a Bunsen burner. Studies showed that there was no loss of ash as a result of the controlled ignition. The charred residue was then ashed in a muffle furnace at 550° C. until constant weight was obtained (about 24 hours). The sample was cooled in a desiccator to room temperature and weighed.

Estimation of glycogen: Analyses were made to ascertain whether the homogenate could be used for glycogen determination and to secure some indication of the amount of glycogen in the carcass. Glycogen was extracted from the homogenates of weanling rats by a modification of the method of Good and associates,[8] and analyses for glucose were done thereon by Nelson's modification of the Somogyi method.[9] Using homogenates equivalent to approximately 2 to 3 Gm. of wet carcass, the glycogen content of one rat was 0.16 per cent (individual values: 0.14 and 0.17) and for the other was 0.21 per cent (individual values: 0.19, 0.22, 0.21). These rats were normal animals which had food available until they were sacrificed as described above.

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*From 20 to 25 Gm. of homogenate had to be used to secure a sample representing 10 Gm. of the wet carcass. This amount could be approximated in 2½ level tablespoonfuls.

†To secure the above weight of sample, 1 to 1½ tablespoonfuls of homogenate were placed in a weighing dish. A piece of aluminum foil was used to cover the crucible during weighing.

Conversion Factor for Calculations.—Aliquots for the various analyses are corrected for the water added during the preparation of the homogenate by dividing the corrected body weight by the total weight of the homogenate. This factor multiplied by the weight of the aliquot taken gives the weight of wet carcass present in the sample (aliquot taken).

Autoclaving.—To determine whether autoclaving had any influence on the proximate analysis of the carcass, tissue homogenates were prepared with and without autoclaving. The tissues were a lean rat without skin, hair, and GI contents, the adipose tissue from a fat rat, and the muscles of a lean rat. Each of the 3 tissues was homogenized in a Waring blendor and then divided into 6 aliquots. Half of the aliquots were autoclaved and the others were not. Analyses for moisture, fat, and nitrogen showed (Table I) no detectable loss as a result of autoclaving.

TABLE I. EFFECT OF AUTOCLAVING ON MOISTURE, FAT, AND NITROGEN CONTENT

TREATMENT	MOISTURE* (PER CENT)	FAT† (PER CENT)	NITROGEN (MG./GM. HOMOGENATE)‡
Autoclaved	77.41 ± 0.04	98.65 ± 0.03	13.91 ± 0.17
Nonautoclaved	77.29 ± 0.14	98.77 ± 0.02	13.93 ± 0.12

The mean and standard deviations are given. Standard deviation $= \sqrt{\frac{\Sigma d^2}{n-1}}$ where Σd^2 is the sum of the deviations from the mean squared and n is the number of analyses.

*Lean rat without skin, hair, and GI contents.

†Adipose tissue of a fat rat.

‡Muscle tissue of a lean rat. (All values represent averages of 3 determinations each.)

TABLE II. INFLUENCE OF WEIGHT OF SAMPLE ON MOISTURE DETERMINATION

NO. SAMPLES	AV. WT. HOMOGENATE	AV. WT. WET CARCASS* (GM.)	AV. WT. DRY CARCASS (GM.)	MOISTURE	
				AVERAGE	STANDARD DEVIATION†
3	1.0971	0.6355	0.4021	36.73	0.29
3	5.0576	2.9294	1.8512	36.81	0.13
3	10.0375	5.8138	3.6721	36.84	0.12
3	23.8116	13.7917	8.7093	36.85	0.08
2	246.3	142.7	90.50	36.58	0.19

*Weight of rat carcass contained in the homogenate sample.

†See footnote to Table I.

Sample Weight.—Several aliquots of the homogenate of an obese rat were dried in a vacuum oven at 70° C. for approximately 45 hours. The values in Table II indicate that the replicability of the results is not influenced by varying the weight of the homogenate from about 1 to 25 Gm. Within this range, increasing the weight of the sample reduces the standard deviation (from 0.29 to 0.08). There is a suggestion of a slight reduction in moisture content when very large amounts of homogenate are used. This is probably due to the difficulty in drying such large samples.

Homogeneity.—In order to determine whether the samples were homogeneous, aliquots were taken from the top and bottom of the container. Before each sample was removed from the jar, the contents were thoroughly mixed by hand shaking. After the samples were taken from the top of the jar, approximately one-half of the homogenate was poured into another container. Duplicate aliquots were taken from the homogenate remaining in the original jar and these were called the bottom aliquots. The results (Table III) show very good agreement for the samples from the top and bottom of the container for moisture, fat, protein, and ash. The above results were obtained from the carcass of an obese rat. Similar results were secured on weanling and adult lean rats.

TABLE III. AGREEMENT OF ANALYTIC DATA SECURED ON DUPLICATES TAKEN FROM THE TOP AND BOTTOM OF THE HOMOGENATES OF AN OBESE RAT
(CORRECTED BODY WEIGHT = 1,235 GM.)

SAMPLE NO.	WEIGHT HOMOGENATE (GM.)	WT. WET CARCASS (GM.)	WT. DRY CARCASS (GM.)	PORTION OF HOMOGENATE	MOISTURE (PER CENT)
A. Moisture Determination					
1T	12.8656	7.6023	5.5035	Top	27.61
2T	13.4095	7.9237	5.7317	Top	27.66
3B	14.2328	8.4102	6.0795	Bottom	27.71
4B	14.7632	8.7236	6.3080	Bottom	27.68
Average					27.67 ± 0.04*

SAMPLE NO.	WEIGHT HOMOGENATE (GM.)	WT. WET CARCASS (GM.)	MG. N/GM. WET CARCASS	PORTION OF HOMOGENATE	PROTEIN† WET BASIS (PER CENT)
B. Protein Determination					
1PT	1.0646	0.6291	13.83	Top	8.64
2PT	0.9967	0.5890	13.92	Top	8.70
3PB	1.0973	0.6484	13.88	Bottom	8.68
4PB	0.9973	0.5893	13.92	Bottom	8.70
Average					8.68 ± 0.03*

SAMPLE NO.	WT. WET CARCASS (GM.)	WT. EXTRACTED FAT (GM.)	PORTION OF HOMOGENATE	FAT WET BASIS (PER CENT)
C. Total Fat Determination				
1T	7.6023	4.7311	Top	62.33
2T	7.9237	4.9378	Top	62.31
3B	8.4102	5.2377	Bottom	62.28
4B	8.7236	5.4295	Bottom	62.24
Average				62.27 ± 0.01*

SAMPLE NO.	WEIGHT HOMOGENATE (GM.)	WT. WET CARCASS (GM.)	WEIGHT ASH (GM.)	PORTION OF HOMOGENATE	ASH WET BASIS (PER CENT)
D. Total Ash Determination					
5T	11.8170	6.9827	0.0800	Top	1.15
6T	10.2916	6.0813	0.0677	Top	1.11
7B	10.5297	6.2220	0.0707	Bottom	1.14
8B	10.0055	5.9123	0.0699	Bottom	1.18
Average					1.15 ± 0.01*

*For standard deviation see footnote to Table I.

†Protein = 6.25 × nitrogen.

TABLE IV CARCASS COMPOSITION OF RATS

NO ANIMALS	AGE (WK.)	SEX	CORRECTED BODY WEIGHT (GM.)	PER CENT OF WET BODY WEIGHT*				H_2O/PROTEIN*	TOTAL RECOVERY* (PER CENT)
				MOISTURE	PROTEIN	FAT	ASH		
6	3	M	39.9 (36.2 – 41.6)†	73.09 ± 0.57	16.30 ± 0.10	6.77 ± 0.47	2.80 ± 0.03	4.49 ± 0.06	98.93 ± 0.09
2	4	M	78.7 (75.3 – 82.1)	73.30 ± 0.18	16.69 ± 0.14	6.28 ± 0.14	2.95 ± 0.02	4.40 ± 0.04	99.21 ± 0.11
4	71	F	318.7 (302.7 – 328.2)	57.79 ± 2.05	17.97 ± 0.27	20.93 ± 2.44	3.11 ± 0.27	3.21 ± 0.11	99.80 ± 0.12
2	71	F	702.6 (578.0 – 827.2)	35.89 ± 1.02	10.68 ± 0.99	51.39 ± 1.98	1.93 ± 0.03	3.38 ± 0.22	99.89 ± 0.07
1	44	M	1235.0	27.67	8.68	62.32	1.15	3.19	99.82

*Mean and standard error determined by Mantel's method[10]
†Range

These data indicate that if the above procedure is used excellent agreement can be secured in analytic results carried out on all samples of the homogenate.

A further measure of the uniformity of the homogenates was secured from an evaluation of the average standard deviations for the analytic data secured to date. The values listed in Table IV represent the averages for a series of 15 rats. The standard deviations for these separate sets of values have been averaged in Table V. The data in this table show that the average standard deviations for moisture, protein, and fat are between 0.10 and 0.20 while that for ash is less than half of these. Although the data listed in Table VI were compiled for another purpose, it does suggest that on the few animals studied, the standard deviations of the different analytic procedures are greater when carried out on the homogenates from lean rats as compared with those from obese rats, the standard deviations in most cases being at least twice as great.

TABLE V. VARIABILITY OF ANALYTIC RESULTS AS AN INDEX OF THE HOMOGENEITY OF THE CARCASS PREPARATION*

PROCEDURE	RANGE FOR PROCEDURE IN PER CENT	AVERAGE STANDARD DEVIATION†	RANGE OF STANDARD DEVIATIONS
Moisture	27.67 – 74.55	0.10	(0.02 – 0.16)
Protein	8.68 – 18.49	0.18	(0.03 – 0.50)
Fat	5.67 – 62.32	0.10	(0.01 – 0.21)
Ash	1.15 – 3.68	0.04	(0.01 – 0.12)

*Results are based on triplicate or quadruplicate analyses on each of 15 homogenate preparations.

†Average standard deviation $= \sqrt{\frac{n_1\ (S.D._1)^2 + \cdots + n_{15}\ (S.D._{15})^2}{n_1 + \cdots + n_{15}}}$ where $(S.D._1)^2$ through $(S.D._{15})^2$ are the standard deviations squared. n_1 through n_{15} are number of degrees of freedom in each group.[11]

TABLE VI. DAY-TO-DAY VARIATIONS IN RESULTS FOR CARCASS ANALYSIS OF AN OBESE (CORRECTED BODY WEIGHT = 827 GM.) AND A LEAN (CORRECTED BODY WEIGHT = 328 GM.) RAT. ALL ANALYTIC VALUES EXPRESSED ON A WET WEIGHT BASIS

RAT NO.	DAY	MOISTURE (PER CENT)*	PROTEIN† (PER CENT)*	FAT (PER CENT)*	ASH (PER CENT)*	TOTAL RECOVERY (PER CENT)
41,164	0	34.87 ± 0.09	9.69 ± 0.07	53.37 ± 0.06	1.89 ± 0.03	99.82
41,164	5	35.06 ± 0.06	9.70 ± 0.15	53.15 ± 0.06	1.90 ± 0.05	99.81
41,155	0	55.68 ± 0.12	18.10 ± 0.44	23.32 ± 0.42	2.73 ± 0.11	99.83
41,155	5	56.09 ± 0.34	17.91 ± 0.16	23.03 ± 0.26	2.77 ± 0.17	99.80

*Mean and standard deviation are given.

†Protein = 6.25 × nitrogen.

Day-to-Day Variations.—Since the analytic results for many methods show considerable day-to-day variation, an estimate of the latter was secured by carrying out complete carcass analyses on two separate days. The homogenates of two rats were analyzed in triplicate or quadruplicate, with the same analyses being repeated 5 days later. The data in Table VI show that the variation in the values for the results secured on different days is no greater than that for the same day.

Carcass Composition of Rats—To indicate the application of the method, data are presented for carcass analyses of 15 rats The results (Table IV) indicate that the sum of the moisture, protein, fat, and ash account for practically all of the animal On this basis, we have accounted for an average of 99 53 per cent of the rat carcass It is likely that a part of the material unaccounted for is glycogen but this is probably no more than 0 2 per cent Should it be desirable to secure exact values for the glycogen content of the carcass, special precautions would have to be taken to minimize the postmortem loss of glycogen

The data show that the percentage of body fat in the rat increases with age whereas the water and protein decrease The ratio of water to protein decreases slightly with age The percentage of ash in the rat's body decreases with age and shows an even greater decrease as the rat becomes obese

DISCUSSION

An experienced technician is able to prepare the homogenates for 6 animals and analyze them in duplicate for the 4 major body components (moisture, fat, protein, and ash) by the above technique in a period of 6 days As far as the homogenates are concerned, from 6 to 8 can be prepared in an 8 hour day In our experience this is considerably faster than is possible with any other method

The homogenate is so uniform that the variation between duplicate results is very small This is especially true for the homogenates of obese and weanling animals

The sum of the values for the 4 major body components for homogenates of carcasses prepared by this procedure is consistently very close to 100 per cent The glycogen content can be determined in the homogenate and, under normal circumstances, would account for an average of about 0 1 per cent and probably no more than 0 2 per cent

The above procedure for preparing carcass homogenates permits duplicate analyses for all methods This is not possible, especially for moisture and fat, by some of the currently available techniques The homogenate can be used in any analytic procedure applicable to tissues, the procedures listed in the paper have been given only because they have been found satisfactory for our work The method of preparing the carcass homogenate should be of value whenever it is desired to determine the carcass content of any compound that is stable to the 15 minutes of autoclaving Animals showing considerable range in body weight can be prepared by the procedure We have successfully prepared homogenates of animals ranging in size from weanling rats to rabbits weighing 2 Kg including guinea pigs and chicks

SUMMARY

1 A method has been devised for the preparation of homogenates of whole animals The procedure consists of autoclaving the animal to soften the skin

and other tissues. Preliminary grinding can be accomplished in a W blendor. Stable homogenates are prepared by putting the blended m through a colloid mill.

2. The homogenate can be prepared in a short time and is so unifo composition that duplicate analyses for moisture, protein, fat, and ash within very small limits. The sums of the values for these carcass compo range from 98.9 to 99.9 per cent for different animals.

3. The procedure is applicable to animals with a considerable ran body weight. It has been used in the preparation of homogenates of a ranging in size from weanling rats to rabbits weighing about 2 kilogram

We wish to thank Dr. William W. Wright of the Food and Drug Administration generosity in making the colloid mill available.

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A RAPID AUTOMATIC SEMIMICRO COLLOID OSMOMETER

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THE measurement of the oncotic (colloid osmotic) pressure of solutions of substances with high molecular weights has long been of interest to workers in biologic and physicochemical fields. The earlier methods of determination have been reviewed by Petit[1] and Briggs.[2] These methods, as a rule, have been very time consuming and have been inapplicable to the rapid measurement of large numbers of samples.

Meehan and associates[3] constructed a colloid osmometer utilizing an unbonded strain gage transducer. The osmometer was found to be impractical, however, due to technical difficulties[4] which included (1) excessive time required to reach equilibrium, and (2) large volume displacement of the system due to movement of membrane. Further work on it was discontinued. Hansen[5] has developed a colloid osmometer which registers, by means of an electric condenser manometer, the balancing pressure which prevents solvent flow across a membrane into the colloidal solution. However, his device requires repeated balancing of the oncotic pressure during each determination with an externally applied hydrostatic pressure from a fluid filled manometer. Rowe[6,7] has constructed an osmometer similar in principle to the one developed by Hansen. It also requires balancing with a variable external hydrostatic counter pressure during the course of each determination. The point of no fluid transfer or balance of oncotic pressure with external hydrostatic counter pressure is determined visually from serial galvanometer readings. The corresponding osmotic pressure is then read on the balancing hydrostatic manometer. The time for each determination is about 40 minutes.[8]

We have developed a fully automatic semimicro colloid osmometer which permits determinations in as little as 5 minutes. This instrument is capable of recording colloid osmotic pressure of small samples (0.5 to as low as 0.1 cm^3). The pressure (negative with respect to atmospheric pressure) produced in a sealed solvent filled chamber by the passage of solvent across a semipermeable membrane into the colloidal solution is recorded by a strain gage. Significant dilution of the colloidal solution by the passage of solvent into it does not occur since the coefficient of volume change with pressure change (dV/dP) for the sealed solvent filled transducer chamber is negligible.

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MATERIALS AND METHODS

The osmometer utilizes a Statham P23D pressure transducer* as the pressure sensing device. The pressure dome of this transducer is a removable plastic fitting that has two standard Luer-Lok male adapters; one on the top center, the other on the side. The orientation of parts of the osmometer is shown in Fig. 1. Figs. 2 and 3 show the assembled instrument.

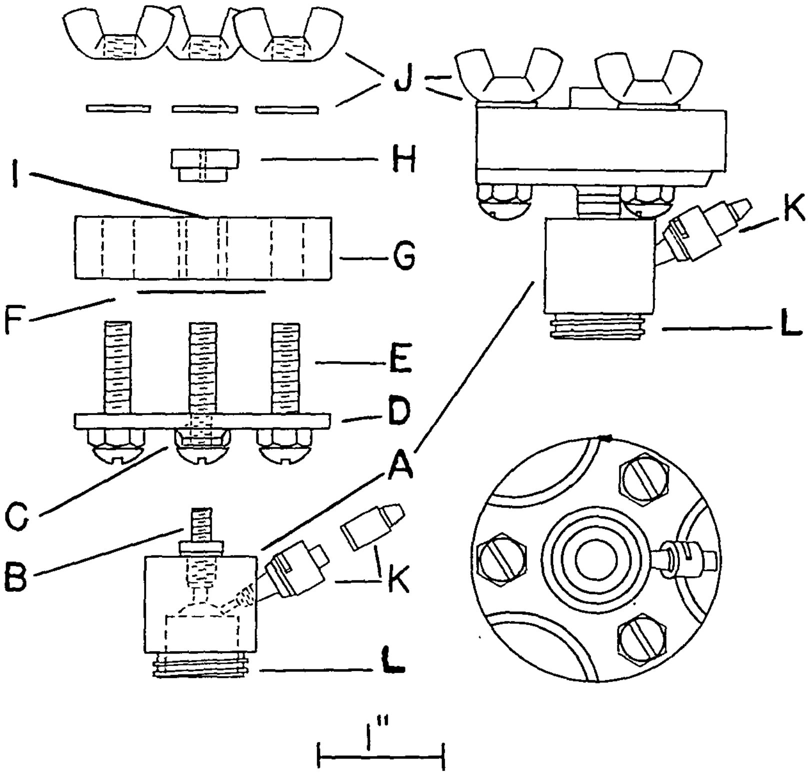

Fig. 1.—Exploded and assembled views of osmometer.

Construction.—The Luer-Lok collar is removed from the top male adapter and the male connection is threaded with an 8-32 S.A.E. thread (Fig. 1, B). A $\frac{1}{8}$ in. thick brass plate, 2 in. in diameter (D), is soldered to a $\frac{1}{8}$ in. thick brass spacer $\frac{1}{4}$ in. in diameter (C). This is then tapped in its center through the spacer and the plate to receive the threaded male adapter (B) from the pressure dome. Three spaces are cut out of the brass plate (D) to provide clearance for the side arm Luer-Lok male adapter (K) on the pressure dome. In addition, 3 tapped screw holes, 120 degrees apart, are placed $\frac{3}{4}$ in. from the center of the brass plate (D) to receive three 12-24 brass screws (E). The brass plate (D) which is to act as a platform on which a semipermeable membrane (F) is to be placed is then screwed tightly to the top threaded male adapter (B) of the pressure dome (A).

Care is taken that the side arm Luer-Lok male adapter of the pressure dome is underlying 1 of the 3 clearance spaces that have been cut out of the brass plate. This provides sufficient working room when the side arm is to be used. The spacer that has been soldered to the brass plate provides sufficient clearance for the side arm as the brass plate is being screwed to the top threaded adapter of the pressure dome. When the plate and pressure dome have been firmly connected, the surfaces of the brass plate (D) and any of the threaded top male adapter (B) that is protruding through the plate are ground and polished on stones and emery cloth of progressive fineness until a highly smooth flat surface is obtained. This surface is to receive the membrane (F).

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*Statham Instruments, Inc., 12401 West Olympic Boulevard, Los Angeles, Calif.

A ¼ in. thick Lucite plate, 2 in. in diameter (G), having a center hole (I) ⅜ in. in diameter is prepared with three ¼ in. diameter clearance holes 120 degrees apart, ¾ in. from the center of the plate. These holes receive the three 12 24 brass screws (E) from the brass plate.

Fig 2.

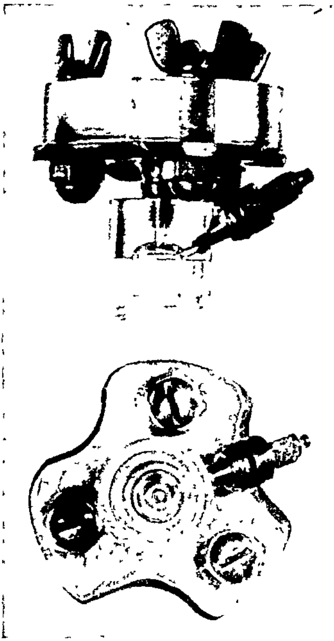

Fig. 3

Figs 2 and 3.—Full size photograph of osmometer

The Lucite plate is then highly polished in the same manner as the brass plate. It serves to hold the membrane on the brass plate and provides a chamber (the ⅜ in. center hole) into which the colloidal solution is placed (I). By varying the diameter of the center hole in the Lucite, chambers of varying capacity may be prepared. The Lucite plate is held in place by wing nuts (J) on each of the 3 screws (E) from the brass plate.

A ¼ in. thick Lucite cover (H), ½ in. in diameter with a center hole 0.5 mm. in diameter, is made for the solution chamber (I). The cover prevents evaporation of colloidal solution within the chamber. The center hole in the cover permits equilibration of pressure within the chamber with atmospheric pressure.

The P23D pressure transducer is powered by a 12 volt storage battery. The electrical output from the transducer is fed into a Statham control box with a heliopot introduced into the zero adjustment circuit. The electrical output from the control box is then connected with a Brown potentiometer recorder with an input sensitivity of 5 mv. full scale.*

The electrical recording system is calibrated directly against pressure by means of a water manometer that is attached to the side arm (K) of the fluid-filled transducer pressure dome. This pressure calibration is done only once since an electrical calibrator on the control box can be used for further calibration once its pressure equivalent in terms of millimeters of water has been determined (Fig. 4).

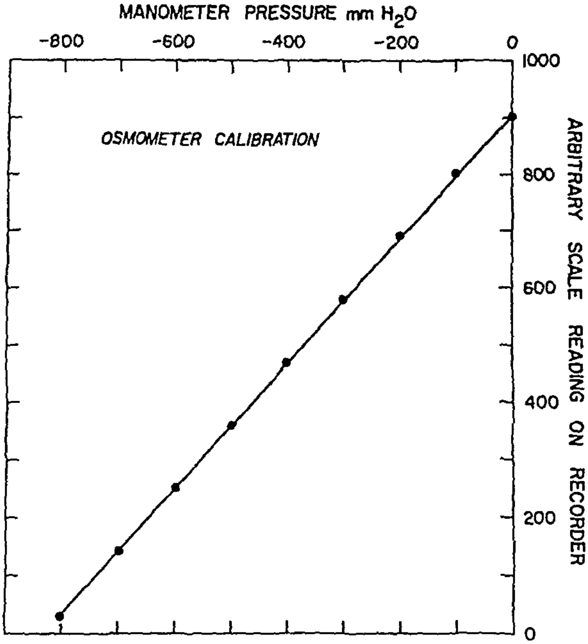

Fig. 4.—Osmometer calibration.

The membranes that we have found satisfactory are commercially prepared.† The 20 mm. diameter size, ultrafine, dense, and very dense, are suitable for the determination of the oncotic pressure of human serum albumin. The membranes can be left in the osmometer and used for many determinations. We have used them for well over 100 runs each and found no leakage of protein across the membrane into the sealed solvent chamber when tested with 10 per cent trichloroacetic or 6 per cent sulfosalicylic acid. In fact, the more often the membrane has been used the more suitable for rapid determinations it becomes since its rigidity and adjustment to the osmometer are improved with use.

Methods.—1. The plastic pressure dome (Fig. 1, A) with attached brass plate (D) is immersed in a solvent bath and filled to the exclusion of all air. Care must be taken that no bubbles persist in the dome. This is easily accomplished by flushing any bubbles with a syringe attached to K.

2. A cap is placed firmly on the Luer-Lok male side arm of the pressure dome (K).

3. The transducer element itself is immersed in the solvent. This can be done with the P23D because the back of the transducer and electrical elements are protected by a plastic jacket extending over the power cord. After all bubbles have been flushed off, the transducer element is placed into position and screwed firmly to the pressure dome at L. This is all done while still immersed in solvent.

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*Model No. Y153x(62)P12-x-(16) was used in these experiments with all 12 leads recording. Calculations were based on the plus marks which are identifiable on the original records.

†Carl Schleicher & Schuell Company, Keene, N. H.

4 The assembled transducer element and attached pressure dome with sealed side arm is then removed from the solvent

5 The membrane (F) is placed on the brass plate (D) over the center hole (I) making sure there are no trapped bubbles underneath it The membrane is kept moist at all times

6 The Lucite plate (G) is placed in position on the brass plate (D) and firmly attached by means of wing nuts (J) placed on the screws (E) protruding from the brass plate

7 The assembled osmometer is now placed in a suitable protecting bag (polyethylene serves well) and immersed in a constant temperature bath

8 After a zero base line has been obtained, the Lucite chamber (I) is cleared of all fluid (best accomplished by filter paper strips) and the sample is placed in the chamber Samples of 0 5 to as low as 0 1 cm^3 can be used with the size chamber described

9 A drop or two of sample is placed on the underside of the small cover of the Lucite chamber (H), taking care not to cover the tiny hole This is done to prevent evaporative loss of solvent from the solution in the osmometer chamber

10 The cover (H) is placed on the sample chamber of the osmometer

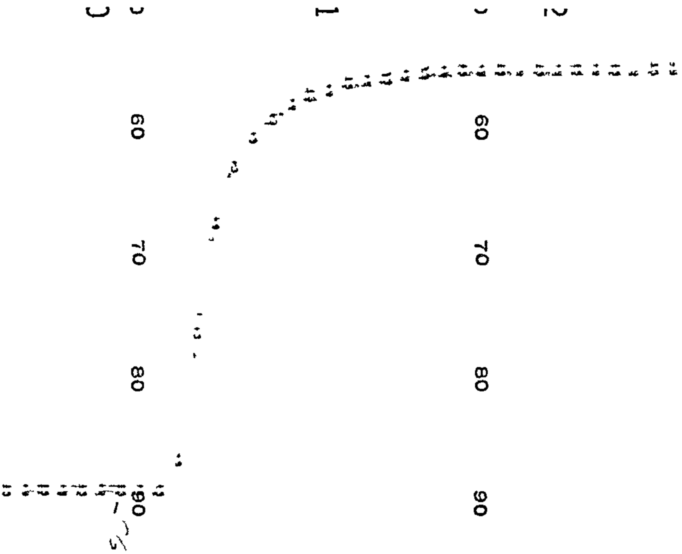

Fig 5—Sample record to approach to equilibrium Record reads from left to right

11 The oncotic pressure of the sample is represented by a plateau in the record (Fig 5) The first determination from the zero base line usually takes about 15 to 20 minutes

12 If serial determinations of solutions with similar solvents are to be run, all that is necessary is to remove the solution from the chamber, flush the chamber a few times with solvent, blot it dry with filter paper, and add the new solution Serial determinations may be accomplished within a few minutes each following the first determination since the membrane has by then become set and adjusted to the osmometer

RESULTS

The standard error of the method based on 8 determinations on human serum was ± 3.5 mm. H_2O from an average value of 345.1 mm.

The approach to equilibrium can be very easily followed since a continuous pressure recording is made (Fig. 5). When the logarithm of the pressure differential from equilibrium ($P\text{-}P_{EQ}$) is plotted against time (t), a straight line is obtained. This is to be expected since the rate of approach to equilibrium (dp/dt) is proportional to the pressure differential from equilibrium $\left[\frac{dp}{dt} = k(P\text{-}P_{EQ})\right]$ (Fig. 6). The same oncotic pressure is obtained when it is approached from pressures either above or below the equilibrium pressure.

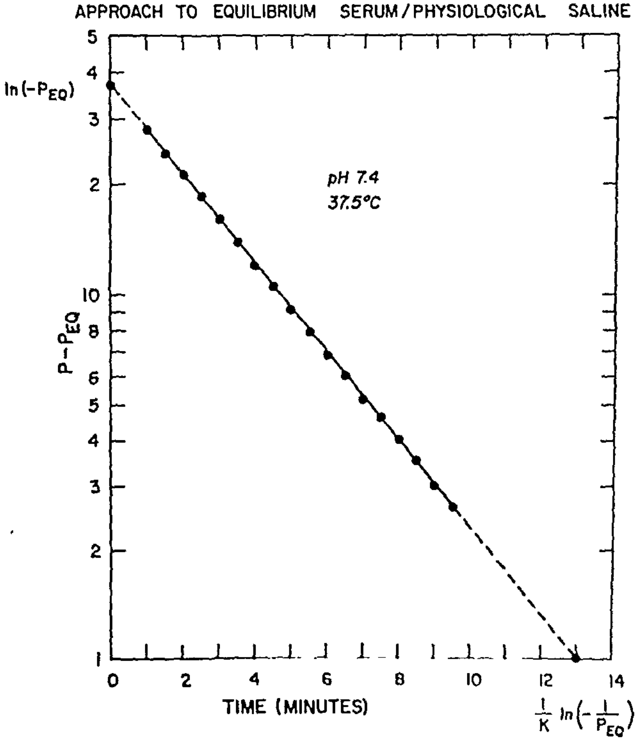

Fig. 6.—Ln approach to equilibrium.

DISCUSSION

This osmometer is particularly suited to serial determinations of oncotic pressure. Once a zero base line has been established with solvent on both sides of the membrane, the first determination will bring the osmometer into a state whereby further determinations can be done rapidly without bringing the instrument back to the zero base line. By starting at a pressure near the oncotic pressure of the sample, much time may be saved during each run.

The amount of solvent transfer across the membrane into the sample during the approach to equilibrium is negligible, even when the determination is started from the zero base line. A demonstration of this negligible fluid transfer is that serial determinations of the same sample of colloidal solution give the same oncotic pressure reading when the run is started from the zero base line as when it is started at a pressure very close or equal to the equilibrium pressure. In addition, solution aspirated from the sample chamber after equilibrium has been reached, gives the same oncotic pressure when it is redetermined starting from the zero base line.

The rate of pressure approach to equilibrium (dp/dt) has been shown to be a function of a rate constant (k) and the pressure differential from equilibrium $(P - P_{EQ})$: $dp/dt = k(P - P_{EQ})$.

Also, dp/dt can be expressed as a function of two other quantities which, in turn, are functions of several other parameters.

A. The rate of transfer of solvent through the membrane $(\delta V/\delta t)_p$

  (1) varies directly with the membrane diffusion constant for the solvent involved,
  (2) varies directly with the membrane area,
  (3) varies inversely with the membrane thickness.

B. The volume elasticity *of the sealed solvent chamber* $(\delta P/\delta V)_t$

  (1) varies directly with the rigidity of the membrane,
  (2) varies directly with the rigidity of the rest of the sealed plastic solvent chamber, however, this factor is negligible compared to the rigidity of the membrane.

$$dp/dt = (\delta V/\delta t)_p\ (\delta P/\delta V)_t = k(P - P_{EQ})$$

Therefore, k depends upon the membrane factors in the following manner:

$$k \propto \left\{ \frac{\begin{array}{l}\text{membrane diffusion constant}\\ \text{membrane area}\\ \text{membrane rigidity}\end{array}}{\text{membrane thickness}} \right.$$

One might suspect that the largest value for k would be obtained by using a very thin membrane with a large surface area exposed to solvent. However, membrane rigidity plays a very important role and the most rigid membrane would be a thick one with a small surface area exposed to solvent.

Meehan and associates[3] attempted to solve the problem of membrane rigidity by using a perforated metal disk to support a thin cellophane membrane with a relatively large surface area. However, even with the disk, the system was not rigid enough to prevent excessive fluid transfer from solvent to sample chamber.

We have found that an unsupported thicker membrane with a small area exposed to solvent provides a rapid and satisfactory arrangement.

When a new membrane is used, there is usually an initial lag in the pressure curve due to the adjustment and settling into position of the membrane as negative pressure begins to develop in the sealed solvent chamber of the transducer.

SUMMARY

A colloid osmometer* has been described with the following advantageous characteristics:

1. It is entirely automatic, i.e., no balancing of oncotic pressure with an externally applied pressure is necessary.

2. A complete pressure-time tracing of the approach to equilibrium is obtained so that the course may be followed during the actual run.

3. Serial determinations may be easily and very rapidly performed.

4. The instrument is capable of semimicrodeterminations on volumes from 0.5 to 0.1 cm.^{3} By making a smaller solution chamber, the volume of solution needed can be made correspondingly less.

We are indebted to Dr. Milton Landowne for keen and perceptive comments regarding the osmometer and to Mr. Raymond Flath for his aid with the electric recording system.

We would also like to thank Dr. Nathan W. Shock and Dr. Bernard L. Strehler for reviewing this manuscript.

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*Patent pending.

QUANTITATIVE BACTERIOLOGIC STUDY OF TISSUES, FLUIDS, AND EXUDATES

Some Simplified Methods

Douglas Lindsey, M D, Dr P H *
Edgewood, Md

IT IS good to know when there is sugar in the urine It is better to know *how much,* and it is best that this knowledge be attained while the information is still of immediate clinical value Except in complex studies, it usually suffices to report the glucosuria on an approximate scale of "trace" to "4+," rather than in milligrams per cent

In comparison with this simple example from clinical chemistry, bacteriology lags behind Clinical bacteriology is still largely a qualitative process, and the time customarily required for making a qualitative determination is more often measured by calendar than by stop watch Pseudoquantitative descriptions, such as "profuse growth" or "predominant organism" give little real information and sometimes can actually be misleading Quantitative bacteriologic determinations can be had, however, at the expense of more time and a considerable laboratory effort There appears to be no commonly used bacteriologic counterpart of the "plus" reading on urine sugar

For many types of organisms of clinical and laboratory importance, it is possible, by the incorporation of a few simple steps into the laboratory regimen, to obtain quantitative bacteriologic readings as a matter of routine These readings will be estimations or approximations, but they certainly can be as useful and as dependable as are "plus" readings for sugar in the urine Also, as long as a presumptive or generic or familial identification of the organism is sufficiently useful, these readings can often be obtained within 12 to 24 hours after the specimen is taken

My experience with the methods to be described is limited to the study of infectious processes in laboratory animals, but there does not appear to be any reason why these methods should not be of clinical value

MATERIALS AND METHODS

Materials—Platinum loop, 0 01 ml (Fisher Scientific Company, No 13 103) Standard cotton tipped swabs (Q tips, Inc, Long Island City, N Y) Pipettes and other standard laboratory glassware

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From the Biophysics Division Chemical Warfare Laboratories Army Chemical Center Maryland and the Division of Surgery Walter Reed Army Institute of Research Washington D C

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*Lieutenant Colonel Medical Corps United States Army Present Address Directorate of Medical Research Chemical Warfare Laboratories Army Chemical Center Maryland

such smears have shown a high degree of internal consistency—progressive increase in estimated concentration with increase of time in progressing infections as well as correlation with clinical indices of severity of infection. They have permitted quantitation of the bacteriologic effect of experimental antibiotic treatment with acceptable standard errors (Table I). In a series of 20 specimens, estimated simultaneously by direct smear and by Petroff-Hauser chamber counts, there was agreement, to the nearest $\log_{10}$, in 18 of the specimens. In a series of replicate smear determinations on 100 specimens the two estimates yielded the same figure in 93 cases. Estimations made on material subjected to successive tenfold dilutions (within the sensitivity range of the procedure) gave the same figure 26 out of 30 times; the successive dilutions were made using the 0.01 ml. loop, starting both with undiluted material and a 10^{-1} dilution made from a swab sample.

TABLE I. EXAMPLE OF DATA OBTAINABLE BY QUANTITATIVE SMEAR ESTIMATES

HOURS AFTER WOUNDING	INFECTED EXPERIMENTAL WOUNDS					
	UNTREATED ANIMALS			*PENICILLIN-TREATED ANIMALS*		
	N	C	S.E.M.	N	C	S.E.M.
9	15	6.93	±0.23	17	5.30	±0.58
16	13	7.62	±0.40	16	7.12	±0.48
25	6	8.66	±0.26	16	7.68	±0.51

N = Number of animals per group.
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By the use of a number of differential and selective media in the plate estimation method, it is possible within 12 to 24 hours to make a "differential" estimate of the flora in the specimen on the order of the following:

> Preliminary culture data indicate the presence in the specimen of the following types of organisms in the approximate per milliliter concentration indicated:

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Preparation of Specimen.—The specimen is used undiluted, or prepared in a dilution which will place the estimated bacterial concentration within the sensitivity range of the smear and plate methods. Liquid specimens (blood or urine) are measured by pipettes; solid specimens are triturated after measurement by volume or weight. Rectal swabs or swabs of wounds can be considered as representing a sample of 0.1 Gm. or 0.1 ml. Most often, we wring out such swabs in a tube containing 0.9 ml. of trypticase soy broth, making a 10^{-1} dilution.

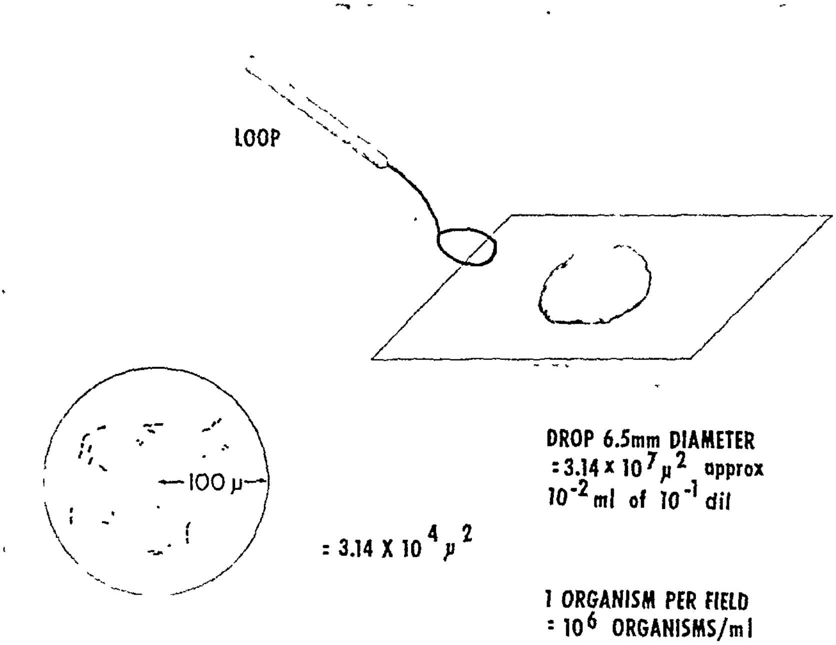
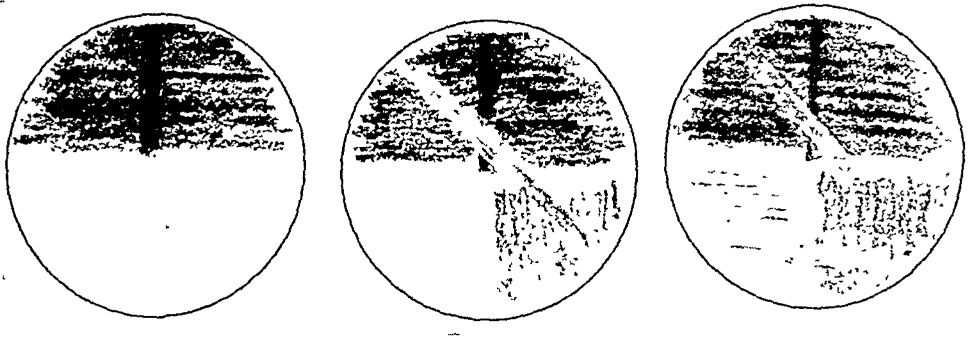

LOOP
$100\ \mu$
$= 3.14 \times 10^4\ \mu^2$
DROP 6.5mm DIAMETER
$: 3.14 \times 10^7\ \mu^2$ approx
10^{-2} ml of 10^{-1} dil
1 ORGANISM PER FIELD
$= 10^6$ ORGANISMS/ml

Fig. 1.—Quantitative smear technique. using drop of standard diameter placed on slide by standard-volume loop.



A. *B.* *C.*

Fig. 2.—Successive steps in serial dilution streaking of single plate for surface plate estimation.

Smears.—One standard 0.01 ml. loopful of prepared material is placed carefully on a glass slide, dried in air without heat, and fixed in 95 per cent ethanol. When the diameter of the microscopic field is known (and it can be determined with sufficient accuracy by use of a bacteriologic or hematologic counting chamber) it is easy to calculate a scale for the number of organisms per average or typical field in relation to the concentration of organisms in the specimen We use a microscope with an oil immersion field approximately 100 μ in radius The loop delivers a drop approximately 3 25 mm in radius For all practical purposes, with a specimen diluted to 10^{-1}, one organism per field represents 10^6 organisms per milliliter in the original specimen (Fig 1)

Plates—One standard 0 01 ml loopful of prepared material is spread evenly over one half of an agar plate 100 mm in diameter (Fig 2, *A*) The loop is flamed and a diagonal streak approximately 5 cm long is made with the flat of the loop through this inoculated half The inoculum so picked up is spread evenly over one of the lower quadrants of the plate (Fig 2, *B*) The loop is again flamed, the inoculated quadrant is passed through with a streak approximately 2 5 cm long, and this inoculum is spread in the remaining quadrant (Fig 2, *C*) After incubation, plates are read quantitatively by a scale such as that shown in Fig 3

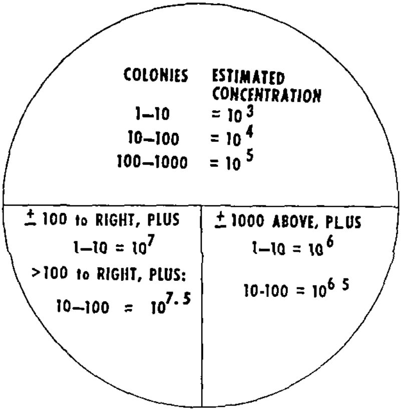


Fig 3—Scale for reading serial dilution streaked plates

RESULTS

By the use of the quantitative smear technique it is possible, within one hour from the time the specimen is taken, to render a report which includes useful quantitative details For example

> A direct smear examination of the specimen indicates the presence of approximately 10^7 gram positive cocci, 10^8 gram positive rods, and 10^8 gram negative rods per milliliter Most of the cocci are morphologically suggestive of fecal streptococci The gram positive rods are about equally divided between large blunt forms in which no spores are seen, and smaller rods which show occasional subterminal oval spores with swelling of the rod The gram negative rods are all minute pleomorphic forms Several degenerated muscle fibers are seen Culture data will be reported

Quantitative estimations on smears are reported only to the nearest $\log_{10}$ of the estimated bacterial concentration *In over 2,000 quantitative estimations*

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50 specimens, all with readings in the lower quadrants of the plates, agreed with the results of parallel pour plate determinations ±0 5 log in 48 instances

TABLE II EXAMPLE OF DATA OBTAINABLE BY SERIAL DILUTION STREAKED PLATE ESTIMATES

HOURS AFTER WOUNDING	INFECTED EXPERIMENTAL WOUNDS TOTAL ANAEROBIC GRAM POSITIVE ORGANISMS	
	UNTREATED ANIMALS	PENICILLIN TREATED ANIMALS
4	3 56 ± 0 24	3 30 ± 0 32
6 25	5 00 ± 0 29	3 45 ± 0 27
9	6 50 ± 0 12	4 70 ± 0 28
12	6 84 ± 0 10	6 20 ± 0 29
16	7 04 ± 0 14	6 40 + 0 16
25	7 15 ± 0 16	6 85 ± 0 12

Twenty five animals in each group Estimates expressed as log$_{10}$ of mean bacterial concentration per milliliter ± standard error of mean

DISCUSSION

The simplicity of both the smear estimations and plate estimations invites ready replication Even in pour plate counting, a few replications makes for greater validity of the estimate than does meticulous or perfect measuring and dilution of the sample[1]

For samples taken by swab, replicate sampling is virtually mandatory A possible exception is in experimental work when a great many determinations are made, and sampling errors can be assumed to "average out", a great deal of the variance in the data in Tables I and II is accounted for by the fact that only single samples were taken

For clinical work it would appear wise to have the swab samples taken by laboratory personnel rather than by the clinician, this is not a matter of getting a "better" sample, but *consistent* samples, so that the currency of the quantitative data will have a stable rate of exchange In the experimental studies for which these methods were developed, sampling times were so scheduled that one individual could make more than 80 per cent of the swabs

So long as estimates are made on a logarithmic basis, the sampling error does not appear to be affected by the usual variations in the quantity picked up by the swab Sanford and associates,[2] have reported the standard swab to pick up "110 ± 22 mg , *depending upon the character of the exudate*" (italics added) In infected experimental wounds in goats we have found the pickup to be 170 mg ± 20 mg (standard deviation) Shortly after wounding the exudate is bloody, and the mean pickup is 170 mg Within a few hours a thin serous exudate appears, the mean pickup drops to 140 mg Later the exudate is seropurulent, with a mean pickup of 170 mg With late and frankly purulent exudates, the mean pickup is 240 mg From a limited number of determinations on bloody exudate containing Cr^{51} tagged red cells,* it appears that the swab *delivers* approximately half of the pickup when it is wrung out in 0 9 ml of broth For logarithmic estimations, it appears sound to accept the range of mean delivery (70 to 120 mg) as resulting in a 10^{1} dilution in 0 9 ml of broth

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II. Total anaerobic gram-positives	$10^{7.5}$
Lecithinase-producing organisms	$10^{7.5}$

The quantities are estimates only to the nearest whole or half $\log_{10}$ (Fig. 3), which explains why they may not appear to "add up": 1×10^6 plus 1×10^6 equals 2×10^6, which is still "10^6" on such a scale. Even with such approximation, this report is sufficient to imply that the specimen, a wound swab, is from a patient suffering from a severe anaerobic infection, probably clostridial.

In more than 9,000 serial dilution streaked plate estimations the method has shown a high degree of internal consistency (Table II). Determinations of

Salt mannitol agar, aerobic, 37° C: total staphylococci, differentiation of presumptively pathogenic organisms[3]

MacConkey's agar, aerobic, 37° C.: total gram negatives, differentiation of coliforms and noncoliforms[4]

SF agar, aerobic, 45.5° C. fecal streptococci

Blood azide egg yolk agar, anaerobic, 37° C: total anaerobic gram positives, differentiation of various types of hemolytic and lecithinitic reactions

We make the SF agar by adding 30 Gm per liter of plain agar to SF broth[5] The blood azide egg yolk agar is based on the medium of Evans[6] Briefly, it consists of an azide blood agar, to which is added 5 per cent by volume of a 1 1 dilution of sterile egg yolk in saline The others are standard media

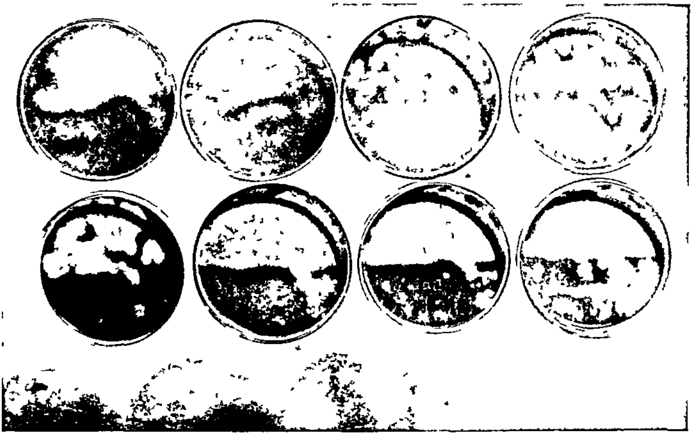

Fig 4—Typical readings on serial dilution streaked plates Blood azide egg yolk medium of Evans, anaerobic incubation Estimations based on lecithinase-positive colonies only Top row (left to right) less than 10^3 (no lecithinase-positive colonies hemolytic streptococci only), 10^3 (single lecithinase-positive colony in upper right of plate) 10^4 10^5 (there is growth in lower quadrants but it is evident at a glance that there are less than 1 000 colonies in the upper half of the plate) Lower row (left to right) 10^6, $10^{6.5}$, 10^7, and $10^{7.5}$

For the method of streaking these plates, to give quantitative estimations at a glance (Fig 4), and on a single plate, of concentrations within a range of some 7 logs ($<10^3$ to $>10^{7.5}$), I am indebted to Baker and Noyes[7] Surface plate counting is not new; methods are well known for distributing a sample by tipping the plate, spreading with a glass rod, or dropping from a pipette.[8] Baker and Noyes demonstrated the simplicity and utility of a standard-volume loop, and the principle which I have come to call serial dilution streaking, to make estimations over a range of 5 logs ($<10^4$ to $>10^6$). The plate estimation method described herein is simply a further extension of their principle, for which they deserve the entire credit.

The principle of placing a known quantity of a known dilution of material on a known area of a slide to permit quantitative determinations on stained

Even when swabs are taken by the same individual, and show close agreement in pickup weight, there is considerable variation between samples. In 100 paired determinations, 56 pairs gave the same reading on the plate scale; 38 differed by one scale reading; 6 differed by two scale readings (1 to 1.5 log).

It is difficult to decide on the basis for calibration of the smear technique; perhaps no calibration is required—the organisms are *there*, they can be seen and counted. The smear technique does not differentiate between viable and nonviable organisms. Plate techniques do not discriminate between single organisms and clumps. For these reasons, smear estimations generally run one log higher than parallel pour plate or serial dilution streaked plate estimations.

For "nearest log" estimations, the dividing line is a decimal scale of "3." If one organism per field represents 10^6 organisms per milliliter in the original specimen, then 3 organisms per field = $10^{6.5}$ organisms per milliliter = "10^7 per milliliter." It is our custom to start the examination in the center of the drop and work out field by field through the edge of the drop, up or down one field, then back to the center for a total of approximately 30 fields. Working with a 10^{-1} dilution, and a field of 100 μ radius, and judging the average or typical field by "sight" and not by actual count, the estimate is made on the following scale:

No organisms seen in
30 fields (i.e., <3 per 100 fields) = $<10^5$
3 per 100 fields to 3 per 10 fields = 10^5
3 per 10 fields to 3 per field = 10^6
3 per field to 30 per field = 10^7
30 per field to 300 per field = 10^8
300 per field to 3,000 per field = 10^9
>3,000 per field = $>10^9$

Calibration of the serial dilution streaked plate method should be performed in the individual laboratory. For estimates made on growth limited to the upper half of the plate, this is a matter of simple arithmetic: a sample of known size and known dilution has been plated. For estimations on the lower half of the plate, a scale such as that shown in Fig. 3 is determined from actual experience in comparison to pour plate determinations. The actual scale will depend on the technique of streaking, the character of the surface of the plates used, and the character of the material streaked, that is, on the efficiency of pickup and distribution of material from the previously streaked area.

The differential and selective media and incubation technique used as a basis for presumptive or generic identifications in rapid quantitative estimations is a matter of choice for the individual laboratory. Our experience has been limited to the following, which are sufficient to support a report of a bacteriologic "spectrum" such as the example cited above.

Blood agar, aerobic, 37° C.: total aerobic estimate.
Blood azide agar, aerobic, 37° C.: total aerobic gram-positives; differentiation of hemolytic reactions without interference from gram-negative overgrowth.

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AN ACCURATE DEVICE FOR THE MEASUREMENT OF DISTANCE ON THE ELECTROCARDIOGRAM AND OTHER PERMANENT RECORDS

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Dearborn and Ann Arbor, Mich.

FOR accurately determining the interrelationship of the events in each of the limb and chest leads of the electrocardiogram, it has long been customary to employ a dual-channel recorder. One of the channels is used for recording each of the limb and chest leads in succession while the other channel is used as a reference lead. The reference lead then serves as a satisfactory time index relative to which the events in the other leads may be measured.

To facilitate electrocardiographic measurement, a number of devices both mechanical and optical have been described. The majority of these consist of rulers or other scales inscribed so as to permit the direct conversion of distance into time.[1] Others consist of caliper-like instruments in which the distance between fixed and movable indicator marks is read on an attached scale.[2, 3] Some of these permit alteration of the scale factor and are adaptable to the use of verniers for increased accuracy. None of these, however, was readily adaptable to the present problem involving the measurement of a large number of simultaneous recordings. This difficulty has been overcome by the device herein described which has been successfully used on a large number of records collected recently by one of us (R. M. P.).

MATERIALS AND METHODS

Method of Construction.—The device consists of an indicator line, the movement of which is registered by an accurate dial indicator; a threaded knob for advancing the indicator line smoothly; and a nonslip base for holding the entire device firmly on top of the electrocardiogram. This is illustrated in Fig. 1.

A dial indicator graduated in units of 0.01 mm. with a total traverse of 25 mm.† was fastened in a convenient position to a stand constructed of $\frac{5}{16}$ inch aluminum stock. Not visible in the figure is a small aluminum support projecting up from the base of the stand to which the lug at the back of the dial indicator was attached by a suitable screw. A bronze bushing $\frac{3}{4}$ inch long was force-fitted into the upright portion of the stand coaxial with the

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actuator rod of the dial indicator The bushing was tapped to receive a $\frac{5}{16}$ 14 threaded brass rod with a knurled knob at one end This threaded rod turns smoothly in the bushing for a distance sufficient to advance the dial indicator the full 25 mm An aluminum turning was drilled to receive the extension of the right hand end of the actuator arm (This extension is available with the dial indicator or can be made from a suitable steel rod) A plastic indicator was attached to the face of the aluminum turning A single line 6 cm. long was inscribed on the underside of the plastic and filled with red pigment to contrast with the ECG This line serves as the indicator Final adjustment of the height of the indicator line was simplified by using slots in the plastic at its point of attachment to the aluminum turning A carrying knob was attached at the right hand end of the stand to facilitate lifting without damaging the actuator rod A sheet of firm rubber, ⅛ inch thick, was cemented to the bottom of the aluminum base

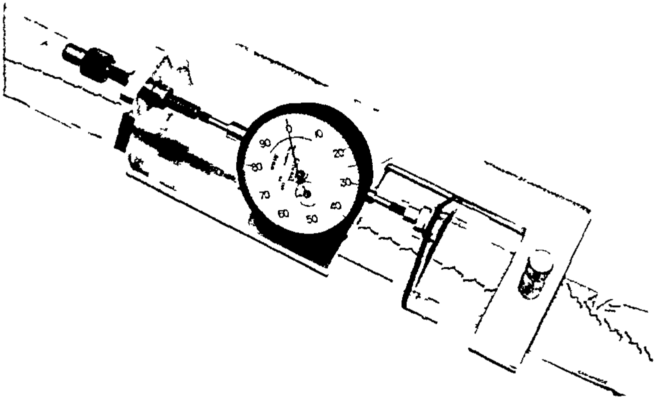

Fig 1

Method of Operation—The instrument is placed on the record to be measured so that the indicator line is parallel to the time line of the record, i e , at right angles to the direction in which the record moved through the recorder Its operation involves only turning the knurled knob to advance the actuator rod of the dial indicator and, with it, the indicator line Distances are then read directly from the face of the dial indicator Note that the face of the dial indicator rotates so that zero may be placed under the pointer for any convenient location of the line The indicator line is long enough to cover both channels of the average dual channel recording and to permit setting the dial at zero with the line correctly located relative to the constant or timing channel

The process of multiplication required to convert the measured distances to time can be simplified by the use of a conversion table or by the use of a dial face graduated in milliseconds

DISCUSSION

This device lends itself well to the measurement of many types of medical recordings including electroencephalograms, electroretinograms, pulse and pressure recordings, and ballistocardiograms It may also find application in the measurement of spectrographic and other photographic plates as well as of paper electrophoresis patterns. If necessary, dial indicators with longer traverse or greater sensitivity may be employed.

Accuracy is limited only by parallax and by the accuracy of the dial ind cator selected. The former is held to a minimum by the location of the indicat line in close proximity to the record being measured. The accuracy of availab dial indicators far exceeds that necessary for the most exacting measurement The type indicator employed here is routinely tested at 0.25 mm. intervals ov its entire range for compliance with the commercial tolerance of plus or min one scale division (0.01 mm.) as specified by the National Bureau of Standards It is clearly more accurate and linear than the most reliable available rule an has proved so in use. In our experience the limiting factor has always been th linearity of the record itself.

SUMMARY

The construction and operation of an instrument for the accurate measur ment of electrocardiograms and other records is described.

We wish to thank the Department of Radiology and, in particular, Norman R. Horwit Wayne State University, for advice and the use of their facilities in the construction of th instrument.

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INFLUENCE OF FUNGUS STRAIN AND LAPSE OF TIME IN EXPERIMENTAL HISTOPLASMOSIS AND OF VOLUME OF INOCULUM IN CRYPTOCOCCOSIS UPON RECOVERY OF THE FUNGI

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THE etiologic diagnosis of a mycotic infection requires the isolation and identification of the fungus. Fungi may be isolated by direct culture or by animal inoculation of pathologic materials. In some instances of proved mycoses, both methods of obtaining cultures may fail.

The difficulties sometimes encountered in obtaining a positive culture led us to investigate two factors influencing the isolation of pathogenic fungi. These were: (1) the effect of increasing the time interval between inoculation of a fungus into mice and sacrifice of the animals for its recovery in culture from the organs, and (2) the influence of increasing the volume of cerebrospinal fluid used for culture on the isolation of *Cryptococcus neoformans* from patients with cryptococcal meningitis.

MATERIALS AND METHODS

Female Swiss white mice (NIH general purpose strain) weighing 17 to 20 grams were used in all experiments. Mice were infected with *Histoplasma capsulatum*, strain 6624, or with *C. neoformans*, strain 1148. Both of these fungi have been used in many experimental studies and have maintained a constant virulence for mice during many serial transfers in culture. However, strain 6624 no longer sporulates freely in culture and may be atypical in other unrecognized characteristics. In later experiments, 3 strains of *H. capsulatum* (F 9961, F 9965, and F 9673) recently isolated from patients in the Clinical Center, National Institutes of Health, and one strain (F 9671) recently isolated from soil on premises where a patient with fatal histoplasmosis had been exposed were converted to the yeast phase and subjected to the same tests.

Yeast phase cultures of *H. capsulatum* were harvested in saline from cysteine blood agar slants after incubation at 37° C. for 48 hours. The suspension was diluted in saline so that each mouse received an inoculum containing 10, 10^2, 10^3, 10^4, 10^5, or 10^6 cells. Hemocytometer counts were used to estimate the number of organisms in the concentrated suspensions. The accuracy of the more dilute suspensions was occasionally checked by culturing in duplicate 1 ml. of the saline suspension on 20 ml. of Sabouraud's agar in a 125 ml. flask.

C. neoformans was harvested in saline from a Sabouraud's agar slant after incubation for 24 hours at 30° C. Dilutions and hemocytometer and colony counts were done in the same manner as for *H. capsulatum*.

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electrophoresis patterns. If necessary, dial indicators with longer traverse or of greater sensitivity may be employed.

Accuracy is limited only by parallax and by the accuracy of the dial indicator selected. The former is held to a minimum by the location of the indicator line in close proximity to the record being measured. The accuracy of available dial indicators far exceeds that necessary for the most exacting measurements. The type indicator employed here is routinely tested at 0.25 mm. intervals over its entire range for compliance with the commercial tolerance of plus or minus one scale division (0.01 mm.) as specified by the National Bureau of Standards.[4] It is clearly more accurate and linear than the most reliable available rule and has proved so in use. In our experience the limiting factor has always been the linearity of the record itself.

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Six weeks after infection 7 of 8 mice still yielded positive cultures from either the liver or spleen, but there was a definite decrease in the number of colonies on each slant.

Only 7 of 36 cultures of liver and spleen taken from 18 mice sacrificed 8 weeks after intraperitoneal infection showed growth of *H. capsulatum*, and these yielded less than 16 colonies per slant. All cultures from animals sacrificed 10 weeks after infection were sterile.

Mice infected intravenously with 10 to 10 cells of strain 6624 showed a similar progressive decrease in the proportion of positive cultures although the decline in recoverable fungi was less striking than seen after intraperitoneal infection.

Results obtained were different when 4 recently isolated strains of *H. capsulatum* were tested under similar conditions. Groups of 10 mice were injected intraperitoneally with 10 , 10^4, or 10^6 cells of each strain. The recorded degree of recovery of Histoplasma from these mice (Table II) is based upon the average colony count in 4 agar slants (2 spleen and 2 liver) from each mouse. In two of the strains, F 9661 and F 9673 there was no observed decrease in the number of colonies growing in cultures with either small inocula or lapse of time up to 10 weeks. In the other two strains, there was a decrease in number of colonies after the lapse of 4 weeks in mice infected with 10^2 and 10^4 cells, although the lower level was sustained at the tenth week. An inoculum of 10^6 cells of F 9671 was lethal and no mice survived for testing at 10 weeks. Similarly, 10^6 cells of F 9665 and of F 9673 killed the last of the mice before the 8 weeks' testing period.

TABLE II. RECOVERY OF 4 TYPICAL, RECENTLY ISOLATED STRAINS OF H. CAPSULATUM AT VARYING TIME INTERVALS AFTER INTRAPERITONEAL INOCULATION AND AT 3 LEVELS OF INOCULA

STRAIN	INFECTING DOSE	2 WEEKS	4 WEEKS	8 WEEKS	10 WEEKS
F 9661	10^2	4+*	4+	4+	4
	10^4	4+	4	4+	4
	10^6	4+	4	4+	4
F 9665	10	4+	3	2	1
	10^4	4+	4+	2+	1
	10	4+	4+	X†	X
F 9671	10^2	4+	3+	3+	3
	10^4	4+	2+	2+	3
	10^6	4+	4	2	X
F 9673	10^2	4	4	4+	4
	10^4	4+	4	4+	4
	10^6	4	4+	X	X

*Key: 4+ represents 50 or more colonies per slant, 3+ 16 to 49 colonies, + 4 to 1 colonies and 1+ 1 to 3 colonies.

†X = no surviving mice for examination.

Since the results with the 4 recently isolated strains did not parallel those observed with the old laboratory strain, all 5 strains were retested simultaneously. Cultures were harvested at 48 hours by suspending cells in cold saline containing 0.1 per cent cysteine. Mice were infected intravenously with 10

Each of the cultures studied was injected in the indicated concentrations intraperitoneally or intravenously into separate groups of 10 mice each. Two animals from each group were sacrificed at various intervals from 2 to 14 weeks following infection. The liver and spleen were removed and a portion of each was placed separately on a Sabouraud's agar slant, crushed with a stiff wire loop, drawn back and forth across the slan several times, and left on the agar. This method of culturing tissues from experimenta mice (rather than the more quantitative method of grinding and diluting weighed portions of tissues) was used because it is the method commonly used in this laboratory for examination of mice inoculated with pathologic tissues or exudates. It was the efficiency of this method which was being tested. All slants were incubated at 30° C. and examined for growth after 1, 2, and 4 weeks.

The effect of increasing the volume of cerebrospinal fluid used in direct cultures on the recovery of Cryptococcus was studied in 7 patients with suspected or proved cryptococcal meningitis. A specimen consisting of 4 to 8 ml. of spinal fluid was cultured in each experiment. The fluid was distributed between test tubes containing 5 ml. of Sabouraud's glucose agar "slants" and Erlenmeyer flasks (125 ml.) containing 20 ml. of agar. Either 0.1 or 0.25 ml. of spinal fluid was pipetted into each tube, and 0.5, 1.0, or 2.0 ml was pipetted into each flask. The tubes were kept semihorizontal until the spinal fluid had dried on the slant. In addition, a 2 ml. sample was centrifuged at 3,000 r.p.m. for 20 minutes and 0.1 ml. of the "sediment" was pipetted in duplicate onto the slants. All tubes and flasks were incubated at 30° C. and were observed for 3 months before being considered sterile.

TABLE I. RECOVERY OF AN OLD LABORATORY STRAIN OF H. CAPSULATUM FROM THE LIVERS AND SPLEENS OF MICE WITH INFECTION OF INCREASING DURATION FOLLOWING INTRAPERITONEAL INOCULATION. AT EACH TIME INTERVAL MICE RECEIVING FROM 10 TO 10^5 CELLS OF H. CAPSULATUM (F 6624) ARE GROUPED TOGETHER SINCE THE RESULTS WERE SIMILAR FOR ALL SIZES OF INOCULA USED

	NUMBER OF COLONIES PER SLANT					TOTAL CULTURES (LIVER AND SPLEEN)
WEEKS*	0	1-3	4-15	16-49	50	
2	2†	0	1	11	22	36
4	4	4	13	8	7	36
6	3	6	4	3	0	16
8	29	5	2	0	0	36
10	16	0	0	0	0	16

*Duration of animal infection prior to sacrifice.

†The two sterile cultures represent the liver and spleen of an animal inoculated with 10 yeast cells.

RESULTS

Recovery of Fungi After Animal Inoculation.—

Histoplasma capsulatum: The results of the first two experiments, using mice infected intraperitoneally with an old, nearly sterile strain of *H. capsulatum* (6624) are summarized in Table I. Mice receiving all sizes of inocula from 10 to 10^5 yeast cells are recorded together in the table, since the final results were generally similar in all groups.

Cultures of liver and spleen were positive in 17 of 18 mice sacrificed 2 weeks after intraperitoneal infection, and the majority showed heavy growth. The cultures from one animal which received an estimated 10 organisms were negative.

of the cultures for 6624 were negative, 96 per cent for F 9665, 58 per cent for F 9661, 35 per cent for F 9671, and 25 per cent for F 9673 were likewise negative. For all 5 strains at all of the 5 sampling periods, 234 of 600 cultures (39 per cent) were negative and 95 of these (40.6 per cent) were from mice inoculated with an estimated 10 cells. Of the 360 cultures made from mice receiving strains F 9661, F 9671, and F 9673, 86 (24 per cent) were negative and 32 of these (37 per cent) were from mice inoculated with an estimated 10 cells.

Recovery of Cryptococcus neoformans: After mouse inoculation no decline in liver and spleen fungal populations with lapse of time was noted following intraperitoneal infection with *C. neoformans*. All cultures of liver and spleen remained strongly positive up to 10 weeks after intraperitoneal infection with 10 to 10^5 cells. There was no evidence that delay in sacrifice of the mice resulted in any change in the number of mice with positive cultures or in the number of colonies per positive slant.

TABLE IV. INFLUENCE OF VOLUME OF SPINAL FLUID CULTURED ON THE PROPORTION OF POSITIVE CULTURES FROM THREE PATIENTS WITH CRYPTOCOCCAL MENINGITIS

PATIENT	NUMBER OF LUMBAR PUNCTURES	VOLUME OF SPINAL FLUID PER TUBE OR FLASK (ML.)	NUMBER OF POSITIVE CULTURES PER TOTAL NUMBER INOCULATED OF CULTURES	COLONIES PER POSITIVE CULTURE
D. K.	3	0.1	1/8	1
		0.25	0/8	–
		0.5	1/4	1
		1.0	3/5	1,3,4
W. G.	5	*0.1 to 0.25*	0/20	–
		2.0	1/2	2
J. N.	4	0.1	0/14	–
		0.25	2/8	1,1
		0.5	1/6	1
		1.0	4/5	1,1,2,2
		2.0	3/5	1,1,3

Recovery of Cryptococcus neoformans From Cerebrospinal Fluid by Direct Culture.—Four of the seven patients studied had many viable organisms per milliliter of spinal fluid as determined by colony counts on Sabouraud's agar. In these patients, as little as 0.05 ml. of spinal fluid gave positive cultures.

In the other 3 patients, the results of culture were influenced significantly by the size of the spinal fluid sample. The results of several spinal fluid studies on each of these patients are summarized in Table IV. The spinal fluid of each of these patients had on the average approximately one viable cell of *C. neoformans* per milliliter, as determined by colony count on Sabouraud's agar. It is clear from Table IV that, when the concentration of viable organisms in the spinal fluid was low, cultures were more likely to be positive if larger volumes of fluid were used. This was true regardless of whether the entire sample was placed in 1 or 2 flasks, or whether it was distributed among many tubes. On several occasions only one colony of *C. neoformans* grew from

10^2, or 10^3 yeast cells of Histoplasma. At autopsy, the spleen was split longitudinally and planted on two slants. A cube of liver approximately 5 mm. on a side was similarly divided between two slants.

Cultures were graded 1 to 4 plus as before. Later, in tabulating results, it was found desirable to estimate the total number of colonies isolated from each mouse and median values were assigned to each of the 3 lower grades (1+ = 2, 2+ = 9, 3+ = 33) and 4+ was valued at 125. Using these estimates, Table III was constructed to show the approximate number of Histoplasma colonies isolated in the entire experiment. Each figure in the Table represents the estimated number of colonies on 8 slants inoculated with spleens and livers of 2 mice.

TABLE III. ESTIMATED TOTAL NUMBER OF COLONIES OF HISTOPLASMA ISOLATED AT THE INOCULATION LEVELS AND AFTER TIME INTERVALS INDICATED. EACH NUMBER REPRESENTS ESTIMATED NUMBER OF COLONIES ON 8 SLANTS INOCULATED FROM SPLEENS AND LIVERS OF TWO MICE

STRAIN	INFECTING DOSE	2 WEEKS	4 WEEKS	6 WEEKS	10 WEEKS	14 WEEKS
6624	10	0	0	0	0	0
	10^2	46	84	132	66	2
	10^3	130	168	17	4	0
F 9661	10	522	28	15	33	8
	10^2	1000	167	324	13	2
	10^3	608	88	30	26	226
F 9665	10	400	88	2	0	0
	10^2	553	40	4	2	0
	10^3	560	383	9	13	2
F 9671	10	345	106	26	6	36
	10^2	246	86	33	6	31
	10^3	415	128	2	57	44
F 9673	10	536	99	147	134	86
	10^2	540	48	168	111	59
	10^3	632	17	79	113	40

In general, in the mice inoculated intravenously, there is a much more apparent decrease in the number of colonies recovered than in mice inoculated intraperitoneally with the same strain of Histoplasma. With the exception of strain 6624, there was a decrease in the number of colonies at the fourth weeks' sampling. However, in 5 of the 15 pairs of mice killed after 6 weeks, there was a rise in the number of colonies. Strains F 9671 and F 9673 and 10^3 cells of F 9661 produced a high level of infection up to 14 weeks. However, at 14 weeks 4 of 15 pairs of mice did not yield cultures. No cultures were recovered from any of the mice inoculated with 10 cells of strain 6624, and these 10 mice are omitted from consideration in the following two paragraphs.

Data from which Table III was derived show that 3.5 per cent of the cultures from mice killed 2 weeks after inoculation were sterile. The number of sterile cultures rose to 22 per cent in 4 weeks, 40 per cent in 6 weeks, 50 per cent in 10 weeks, and 59 per cent in 14 weeks.

Large differences in ability to survive and to produce chronic infections in mice were noted among the 5 strains tested. Thus, at 14 weeks, 94 per cent of the cultures for 6624 were negative, 96 per cent for F 9665, 58 per cent for F 9661, 35 per cent for F 9671, and 25 per cent for F 9673 were likewise negative. For all 5 strains at all of the 5 sampling periods, 234 of 600 cultures (39 per cent) were negative and 95 of these (40.6 per cent) were from mice inoculated with an estimated 10 cells. Of the 360 cultures made from mice receiving strains F 9661, F 9671, and F 9673, 86 (24 per cent) were negative and 32 of these (37 per cent) were from mice inoculated with an estimated 10 cells.

Recovery of Cryptococcus neoformans. After mouse inoculation no decline in liver and spleen fungal populations with lapse of time was noted following intraperitoneal infection with *C. neoformans*. All cultures of liver and spleen remained strongly positive up to 10 weeks after intraperitoneal infection with 10 to 10^6 cells. There was no evidence that delay in sacrifice of the mice resulted in any change in the number of mice with positive cultures or in the number of colonies per positive slant.

TABLE IV. INFLUENCE OF VOLUME OF SPINAL FLUID CULTURED ON THE PROPORTION OF POSITIVE CULTURES FROM THREE PATIENTS WITH CRYPTOCOCCAL MENINGITIS

PATIENT	NUMBER OF LUMBAR PUNCTURES	VOLUME OF SPINAL FLUID PER TUBE OR FLASK (ML.)	NUMBER OF POSITIVE CULTURES PER TOTAL NUMBER INOCULATED OF CULTURES	COLONIES PER POSITIVE CULTURE
D. K.	3	0.1	1/8	1
		0.25	*0/8*	–
		0.5	1/4	1
		1.0	3/5	1,3,4
W. G.	5	0.1 to 0.25	0/20	–
		2.0	1/2	2
J. N.	4	0.1	0/14	–
		0.25	2/8	1,1
		0.5	1/6	1
		1.0	4/5	1,1,2,2
		2.0	3/5	1,1,3

Recovery of Cryptococcus neoformans From Cerebrospinal Fluid by Direct Culture.—Four of the seven patients studied had many viable organisms per milliliter of spinal fluid as determined by colony counts on Sabouraud's agar. In these patients, as little as 0.05 ml. of spinal fluid gave positive cultures.

In the other 3 patients, the results of culture were influenced significantly by the size of the spinal fluid sample. The results of several spinal fluid studies on each of these patients are summarized in Table IV. The spinal fluid of each of these patients had on the average approximately one viable cell of *C. neoformans* per milliliter, as determined by colony count on Sabouraud's agar. It is clear from Table IV that, when the concentration of viable organisms in the spinal fluid was low, cultures were more likely to be positive if larger volumes of fluid were used. This was true regardless of whether the entire sample was placed in 1 or 2 flasks, or whether it was distributed among many tubes. On several occasions, only one colony of *C. neoformans* grew from a total sample of 4 ml. of spinal fluid. In none of the 3 patients was the organism isolated by culturing a total of 0.2 ml. of "sediment" from 2 ml. centrifuged at 3,000 r.p.m. for 20 minutes.

DISCUSSION

Previous studies by Salvin[1] and quantitative tests by Rowley and Huber[2] indicated that tissue titers were maximal 14 days after infection with *H. capsulatum* and progressively decreased thereafter. Our studies confirm these observations and demonstrate differences among strains of Histoplasma and one fallacy of using old atypical laboratory strains of fungi in investigations of this type.

When infected tissues or body fluids from patients suspected of *H. capsulatum* infection are injected intraperitoneally into mice, the time interval between animal injection and sacrifice of the mice for culture may be of great importance. In the present studies, best recovery of fungi was obtained with one old laboratory strain and two more recently isolated strains when the animals were sacrificed 2 weeks after infection. With these strains, as the duration of time following animal inoculation was increased, the number of animals demonstrating infection, or the number of colonies isolated from infected mice decreased, suggesting that mice spontaneously eliminate or markedly diminish *H. capsulatum* infections. Two other strains possessed a greater ability to survive and produce a chronic infection in the mouse. No decline with time in isolations of fungi was noted in experimental *C. neoformans* infections.

It is suggested that in diagnostic studies several mice be sacrificed 2 weeks after intraperitoneal injection of tissues or body fluids where *H. capsulatum* infections are suspected. This would provide optimum conditions for the isolation of some strains of *H. capsulatum* and would also be satisfactory for the recovery of *C. neoformans*. The optimum time of sacrifice for isolation of other pathogenic fungi (including other strains of *H. capsulatum*) has not been adequately studied. Until this time is known, additional mice should be sacrificed 4 to 8 weeks after injection.

When mice are inoculated with soil suspensions in studies of saprophytic sources of Histoplasma and other pathogenic fungi, other cogent considerations may dictate a longer time interval between inoculation and sacrifice. Saprophytic bacteria and fungi present in such inocula may survive for some time. Unless mice are allowed to live until such saprophytes have been destroyed, growth of pathogens may be inhibited or obscured. Therefore, an interval of 4 to 6 weeks between inoculation and sacrifice is customarily allowed as an acceptable compromise in studies of this type.

Three of the 7 patients with suspected or previously proved cryptococcal meningitis had negative spinal fluid cultures when small volumes were used for direct culture. In each case, Cryptococcus was isolated when the size of the sample used for culture was increased. It is probable that the value of culturing large samples applies equally well to the isolation of other species of fungi and also to other body fluids and exudates.

Therefore, when conventional cultures for fungi are negative in a patient suspected of harboring a mycotic infection, it is suggested that larger volumes of fluid be cultured. As the size of the sample is increased it is important to increase the volume of culture medium used since a high blood or exudate to medium ratio may inhibit the growth of some microorganisms. At least 20 ml. of blood, urine or exudate and a minimum of 5 ml. of spinal fluid should be used in order to increase the likelihood of obtaining positive cultures.

SUMMARY

The present experiments show that the time interval between inoculation and sacrifice was important in the recovery of some strains of *H. capsulatum* from liver and spleen of mice used in experimental studies. The optimum interval was 2 weeks. Increasing the interval to 8 to 14 weeks decreased the proportion of positive cultures of certain strains. A uniform decrease was not apparent in some recently isolated strains. No decrease in positive cultures was noted upon increasing the interval between inoculation and sacrifice after infection with *C. neoformans*. When mouse inoculation is used as a method of *diagnosing suspected fungal disease, it is suggested that several mice be sacrificed* 2 weeks after intraperitoneal injection of specimens possibly infected with fungi, and that additional mice be sacrificed 4 to 8 weeks after inoculation.

Evidence is also presented indicating that culture of a large volume of spinal fluid is an important factor in the isolation of *C. neoformans* from some patients with cryptococcal meningitis. It is suggested that at least 20 ml. of *blood, urine or exudate, and a minimum of 5 ml. of spinal fluid be cultured* when conventional cultures for fungi are negative in a patient suspected of harboring a mycotic infection.

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A PRACTICAL METHOD FOR THE SEPARATION OF LYMPHOCYTES FROM GRANULOCYTES

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Many methods have been described for the isolation of leukocytes from whole blood, as well as for the separation of peripheral blood lymphocytes and granulocytes.

Jago[1] has recently reviewed the literature so that it is only necessary to mention a few of the more important and latest contributions.

Kline[2] modified Ottesen's method and used a "Lucite swimmer." Following centrifugation, a well-defined layer of cells, containing the lymphocytes, is present within the "swimmer's" central capillary. Careful aspirations resulted in lymphocyte fractions above 90 per cent in purity.

Jago[1] described a method based on the differing densities of lymphocytes and granulocytes and employing sedimentation and centrifugation. In her experiments the mean percentage yield of lymphocytes was 32 ± 22, the mean percentage purity of lymphocytes 99 ± 2.2.

Philippu[3] used the method described by Szilard and separated the different types of leukocytes by centrifugation of the leukocyte suspension in a Wintrobe tube. He reported a top layer consisting of 92 to 96 per cent lymphocytes and a bottom layer of 90 to 98 per cent granulocytes.

This laboratory has long been engaged in the studies of leukocyte physiology.[4-6] As part of these investigations, it has become important to separate lymphocytes and granulocytes of the peripheral blood with a sufficient yield to enable us to perform desoxyribose nucleic acid (DNA) extractions. Accordingly, a simple method has been developed which allows good separation and yield.

MATERIALS AND METHODS

Methods.—Initial separation of W.B.C. from R.B.C. is performed with the use of a dextran sedimentation method as mentioned in the articles by Skoog and Beck.[7, 8] Lymphocytes and granulocytes, differing in density, are then separated by centrifugation.

Blood is withdrawn in siliconized syringes from the jugular vein of a dog. Twenty-five milliliter aliquots of this blood are added to 10 ml. of 6 per cent dextran[*] in saline and 0.5 ml. of 5 per cent Sequestrene[†] in distilled water as an anticoagulant. Following sedimentation of the agglomerated erythrocytes, which takes approximately 20 to 25 minutes at room temperature, the supernatant containing the leukocytes is pipetted into 50

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[*] Dextran with an average molecular weight of 228,000; supplied by the R. K. Laros Company, Bethlehem, Pa.

[†] Sequestrene disodium (disodium-ethylenediaminetetraacetate dihydrate), Alrose Chemical Company, Providence 1, R. I.

ml. centrifuge tubes. This is then centrifuged for 30 minutes at 1,800 to 2,000 r.p.m. in a size 2 International centrifuge in a coldroom at 4° C.

The supernatant plasma dextran solution is carefully withdrawn to approximately 0.5 cm. above the sediment. All sediments are pooled together and resuspended in the plasma dextran supernatant or normal saline so that a final suspension of 40,000 to 50,000 leukocytes per cubic millimeter is obtained.

Fig. 1.—Lymphocyte granulocyte separation in plastic tube. Top white layer is lymphocyte rich, middle layer is granulocyte rich, and bottom layer contains red cells with very few leukocytes.

The concentrated leukocyte suspension is centrifuged in plastic tubes especially de*veloped for that purpose. Tygon tubing,* outer diameter ¼ in., inner diameter $\frac{3}{16}$* in., and wall thickness $\frac{1}{32}$ in., is cut into 5 in. lengths and sealed at one end with a Dobeckmun Super Sealer.† These plastic tubes with a capacity of 2 ml. are filled with the suspension by means of a Wintrobe hematocrit pipette. To prevent bending during centrifugation, the plastic tubes are placed within small Lusteroid tubes.

After centrifugation for 15 minutes at 1,800 to 2,000 r.p.m., the sediment shows 3 distinct layers (Fig. 1). The top layer is white and contains lymphocytes and platelets. The second layer is pink and granulocyte rich. The bottom layer, dark red in color, consists mainly of erythrocytes. The layers are separated by the application of surgical hemostats and cut with a razor blade at the junction of the layers.

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*Tygon tubing is a product of U. S. Stoneware Company, Plastic & Synthetic Division, Akron, Ohio.

†Dobeckmun Super Sealer, Dobeckmun Company, Cleveland 1, Ohio, and Berkeley 2, Calif.

During the separation procedure, leukocyte and differential counts were done on the various supernates and cell suspensions. Volumes were recorded in order that percentage purity and yields could be calculated.

TABLE I. RESULTS OF LYMPHOCYTE-GRANULOCYTE SEPARATIONS

Dog No.		100-131	82-132	157-142	SUMMARY
No. of separations		12	12	1	25
Mean per cent yield of leukocytes		86.9	78.4	90.2	82.9
Range		67.4-100	64.5-93		64.5-100
Lymphocyte-rich samples	Mean per cent yield of lymphocytes from whole blood	77.1	53.6	100	66.7
	Range	24.8-100	15.2-100	–	15.2-100
	Mean per cent yield of lymphocytes from supernatant	83.8	64.5	76.4	74.3
	Range	39.2-100	32.8-100	–	32.8-100
	Mean per cent purity of lymphocytes	72.6	68.8	62	70.4
	Range	66-82	42-85	–	42-85
	Mean per cent granulocyte contamination	25.3	23.8	38	25.1
	Range	16-31	11-50	–	11-50
Granulocyte-rich samples	Mean per cent yield of granulocytes from whole blood	69.8	68.1	31.3	67.4
	Range	44.9-90.1	48.3-88.2	–	31.3-90.1
	Mean per cent yield of granulocytes from supernatant	76.8	86.8	38.6	80.1
	Range	55.8-96.5	71-9-100	–	38.6-100
	Mean per cent purity of *granulocytes*	76.4	74.8	79	75.7
	Range	58-86	66-83	–	58-83
	Mean per cent lymphocyte contamination	23.3	18.9	21	21.1
	Range	13-38	11-26	–	11-38

RESULTS AND DISCUSSION

The results obtained in 25 separations are shown in Table I.

Many of the methods for lymphocyte-granulocyte separation described in the literature result in a high degree of purity of particular cell preparations. However, the yield of lymphocytes is usually quite poor. The most recently reported technique[1] resulted in a mean per cent yield of lymphocytes of 32 ± 22. With the method described in this paper, the mean per cent yield of lymphocytes was 66.7.

Another advantage of this method is that it is possible to reinfuse the erythrocytes after saline washing. This becomes an important consideration when the test animal must be subjected to large and multiple bleedings.

Since this method was developed in the course of DNA P^{32} studies in which the DNA is extracted from white cells employing the Simmons technique,[5] platelets containing little or no DNA present no problem. Similarly the minimal erythrocyte contamination is of no consequence.

Following the determinations of DNA P^{32} specific activity for the two cell fractions and, knowing the differential counts, lymphocyte and granulocyte DNA P^{32} activity are then readily calculated using simultaneous equations.

The use of the plastic Tygon tubes for the final step of the separation has the advantage of increased yield of the cell fractions, reduced contamination by cells from adjoining layers, and diminished loss of cells with handling. Ingram and associates[9] have also employed flexible plastic tubing for the concentration of leukocytes from small samples of blood.

Smears of the various fractions demonstrate that morphologically there appears to be little or no change in cell structure. A slight increase in disintegrated cells is usually noted.

SUMMARY

1. A method is described for lymphocyte-granulocyte separations of the peripheral blood of dogs.

2. Leukocyte suspensions are obtained from whole blood with a modification of the dextran method. The leukocytes after suitable resuspension are separated into lymphocyte-rich and granulocyte-rich fractions by centrifugation in special plastic tubes.

3. In 25 separations in a test series of 3 dogs, the mean percentage yield of lymphocytes was 66.7, with a mean percentage purity of lymphocytes of 70.4. The mean percentage yield of granulocytes in the granulocyte-rich specimen was 67.4, with a mean percentage purity of granulocytes of 75.7.

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PREPARATION OF A HUMAN PLASMA FRACTION RICH IN PROTHROMBIN, PROCONVERTIN, STUART FACTOR, AND PTC AND A STUDY OF ITS ACTIVITY AND TOXICITY IN RABBITS AND MAN

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It has been known since the work of Quick in 1937[1] that the intravenous injection of normal rabbit plasma transitorily corrects the prolonged prothrombin time of rabbits that have been fed spoiled sweet clover hay, and that no such correction is obtained following the injection of normal rabbit serum. Since the identification of the toxic principle in spoiled sweet clover hay as a coumarin compound by Stahmann and associates[2] in 1941, others[3, 4] have raised the prothrombin level of coumarinized animals by the injection of prothrombin-rich animal plasma fractions.

Recently the intravenous administration of human plasma fractions rich in antihemophilic factor B (AHF-B, PTC)[5, 6] or proconvertin[7, 8] has been reported in patients congenitally deficient in one or the other of these factors essential for normal hemostasis. The present paper describes (1) the preparation of a human plasma fraction hereinafter referred to as PPB, containing prothrombin, the proconvertin complex (see below), and AHF-B (PTC) in high concentration, (2) its intravenous administration to rabbits made deficient in all these factors by prior administration of a coumarin derivative, and (3) the results of preliminary trials of this product in man. Such a fraction could have therapeutic value not only in the conditions in which vitamin K_1 is of no value such as congenital single deficiencies of these factors[9-16] and in acquired combined deficiencies occurring in hepatocellular insufficiency,[17] but also in conditions in which parenteral vitamin K_1 has a beneficial effect (coumarin drug overdosage,[18] biliary obstruction,[17] and prolonged antibiotic therapy[17]). A plasma fraction would raise the levels of the factors in question immediately whereas the vitamin K_1 effect is delayed.

MATERIALS AND METHODS

Thromboplastin was an acetone extract of human brain; buffer was veronal acetate, pH 7.4. All coagulation factors were assayed by methods previously described by one of us with M. J. Larrieu.[19, 20] What was formerly called "specific assay for proconvertin" utilizing Seitz-filtered bovine plasma as substrate plus calcium and tissue thromboplastin

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will hereafter be referred to as "proconvertin complex" assay, for it has become apparent[17] that a deficiency of proconvertin (factor VII, SPCA [illegible]) *or* of Stuart factor will give a prolonged clotting time using this substrate.

Stuart factor was assayed by our modification of Bachmann's[22] method: to 0.1 ml. bovine plasma filtered once through a 20 per cent asbestos pad (J. C. Carlson Ltd.) is added 0.1 ml. of the plasma or fraction to be tested diluted 1:10 in buffer, 0.1 ml. cephalin[23] diluted 1:30, 0.1 ml. Russell viper venom (Stypven, Burroughs Wellcome) diluted 1:50,000 and 0.1 ml. 0.025 M CaCl. Stypven concentrations up to 1:10,000 were also found satisfactory. Cephalin and Stypven dilutions were stable at +1° C. for at least one week. Independently a similar test has recently been developed by Sise and associates.[24] Our modification includes the use of the mixed soybean phosphatide preparation Asolectin (Associated Concentrates), 0.1 per cent in buffer, instead of cephalin, as Asolectin appears more stable on storage. Nine month old plasma (stored at −20° C.) from M. M., a patient with known congenital Stuart factor deficiency,[25] kindly supplied by Dr. J. Bernard and J. Caen and studied in this laboratory by Dr. M. J. Larrieu, clotted in 90 seconds in this assay system, whereas fresh plasma from Y. G., a patient with known congenital proconvertin deficiency previously reported by one of us,[6] clotted in 20 seconds as did fresh or stored normal plasma.

The plasma of Y. G. was also used as substrate in a one stage test of the content of proconvertin in some fractions. For calculation of percentages of all factors appropriate dilutions in buffer were tested. Protein determinations were done by the biuret method.

Preparation of Fraction PPB.—Ten lots were made by the following procedure which, with minor variations, proved to be the most satisfactory. 400 ml. of blood from each fasting donor were collected under sterile conditions through a plastic column containing 70 Gm. of the cation exchange resin Amberlite IR 120 in the sodium cycle and into silicone coated 500 ml. bottles containing 10 ml. of 0.01 to 0.02 M trisodium citrate. The bottles were then centrifuged at +5° C. and 2,600 r.p.m. for 30 minutes. The erythrocytes were ready for therapeutic use as "packed cells" as soon as the plasma was sterilely suctioned off into 500 ml. bottles containing an amount of tricalcium phosphate (Baker, "Analyzed Reagent") such that the latter's concentration in plasma was 0.3 to 0.6 per cent. This adsorbant was chosen as having the highest absorptive capacity per unit weight of several tested (tricalcium phosphates BLB, Fisher, Merck, and Prolabo, barium sulfates Merck and Prolabo). Adsorption was performed by mechanical to and fro agitation of these bottles for 10 minutes at room temperature. The bottles were then centrifuged at +5° C. and 2,600 r.p.m. for 30 minutes. The adsorbed plasma was decanted and used as source for the preparation of sterile fibrinogen and AHF A. The adsorbent was washed once with a volume of normal saline equal to that of the original plasma, and the wash was discarded after centrifugation at 2,600 r.p.m. for 30 minutes. Elution was carried out at room temperature on a magnetic agitator for 15 minutes, the eluant being 1/20 of the original plasma volume of 0.18 M trisodium citrate at pH 8.0. These appeared to be the optimal molarity and pH values, although elution was quite satisfactory with 0.05 to 0.3 M trisodium citrate at pH 5 to 10. Alternatively, two elutions with 1/40 of the plasma volume were pooled, thereby increasing the yield of coagulation factors eluted.

After centrifuging (International refrigerated centrifuge) at 4,000 r.p.m. for 30 minutes, the eluate, which was a clear pale yellow solution, was further concentrated and cleared of its high citrate content by dilution with an equal volume of distilled water and precipitation at pH 5.2 from a final ethanol concentration of 25 per cent at −5° C. The precipitate, after centrifugation at −5° C., was resuspended in 0.1 M glycine containing 1/10 volume 0.1 M trisodium citrate, the pH was adjusted to 7.0 to 8.0 and the fraction was shell frozen and lyophilized. Such lyophilized products did not develop thrombic activity over 5 months' time. Prothrombin concentrations were 110 to 220 times that of normal

PREPARATION OF A HUMAN PLASMA FRACTION RICH IN PROTHROMBIN, PROCONVERTIN, STUART FACTOR, AND PTC AND A STUDY OF ITS ACTIVITY AND TOXICITY IN RABBITS AND MAN

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PARIS, FRANCE

IT HAS been known since the work of Quick in 1937[1] that the intravenous injection of normal rabbit plasma transitorily corrects the prolonged prothrombin time of rabbits that have been fed spoiled sweet clover hay, and that no such correction is obtained following the injection of normal rabbit serum. Since the identification of the toxic principle in spoiled sweet clover hay as a coumarin compound by Stahmann and associates[2] in 1941, others[3, 4] have raised the prothrombin level of coumarinized animals by the injection of prothrombin-rich animal plasma fractions.

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had received a total of 75 to 100 mg of BL 5 orally during the first 5 days. Volumes of fraction injected, 1 to 2 ml, were calculated to bring the rabbit plasma levels up to approximately 100 per cent in prothrombin. Oxalated blood samples were obtained from the ear veins. The blood sample collected 10 minutes after the injection of PPB was drawn from the opposite ear from that into which the fraction was injected.

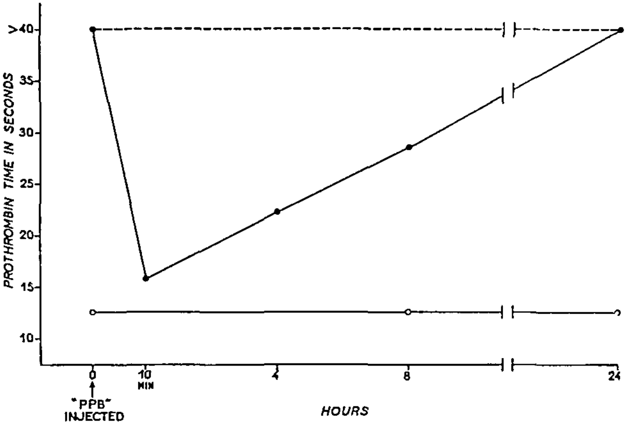

Fig. 1.—Effect of intravenous injection of "PPB" on Quick's prothrombin time of rabbits which have received BL 5 (cyclocumarol). Average of results in 8 injected rabbits. Broken line: uninjected rabbits having received BL 5 alone. Open circles: uninjected rabbits having received no BL 5.

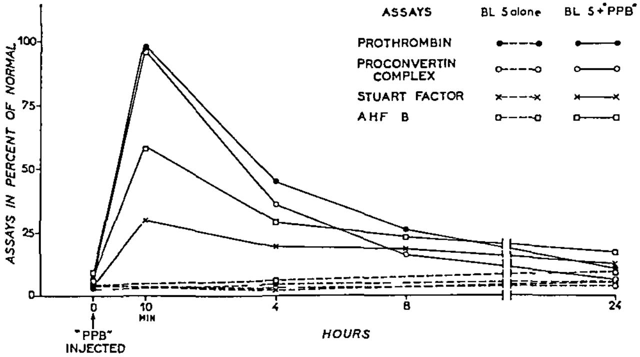

Fig. 2.—Prothrombin, proconvertin complex, Stuart factor, and AHF B in same experiment as Fig. 1.

Figs. 1 and 2 show the effect of the injection of PPB on the prothrombin times (Quick's one stage method) and the levels of prothrombin, proconvertin complex, Stuart factor, and AHF B. The average of all results is represented. At 10 minutes following injection, the

Hoag and associates[7] recently reported the intravenous effect of highly concentrated (49 to 88 times normal plasma by volume) human proconvertin (plus Stuart factor?) on 4 patients with congenital hypoproconvertinemia. This product was made from $Al(OH)_3$ adsorption of outdated plasma. Their survival data in man suggest a considerably shorter half-life during equilibrium (75 to 108 minutes) than those presented here (50 to 55 hours).

Frick[37] studied the disappearance curve of stable factor (proconvertin) in man following a single large dose of Warfarin (3-[α-acetonylbenzyl]-4-hydroxycoumarin), and following infusion of normal plasma in individuals whose stable factor was severely depressed by prior administration of Warfarin. He concluded that the half-life of stable factor in normal man ranges between 5.5 and 10.8 hours. The assay system used is probably sensitive to both proconvertin and Stuart factor, however.

The administration of blood, plasma, or serum to patients with deficiencies of one or more of the factors in PPB has generally been disappointing[14, 29-33]; 600 ml. of fresh whole blood given to a series of adults with chronic severe deficiency of prothrombin and proconvertin had almost no effect on the levels of these factors tested during and at intervals following infusion.[34] Results of such injections are in accord with data presented here concerning the rapid disappearance of the injected factors from the circulating blood. High levels of injected factors cannot easily be obtained, short of an exchange transfusion procedure, unless the injected factors are in concentrated form.

To our knowledge, no commercial product exists containing concentrated human prothrombin, proconvertin, Stuart factor, and AHF-B. Behring Werke (Marburg, Germany) markets ACC-76, which is prepared from human plasma and said to be rich in convertin and accelerin. A lot tested by us, dissolved according to instructions, had little proaccelerin activity and 9 times the proconvertin activity of normal plasma. Its effects in congenital hypoproconvertinemia have been definite, although minimal.[8, 35]

It is possible that human plasma fraction PPB contains the virus of homologous serum jaundice if it was present in the pooled plasma used as source material. Until this question is clarified, such a fraction should be used with caution and the size of the plasma pools kept to a minimum.

SUMMARY

1. A method for preparing a human plasma fraction rich in prothrombin, proconvertin (SPCA precursor, factor VII), Stuart factor, and AHF-B (PTC) is described. Final yields after lyophilization were 46 to 99 per cent, being highest for prothrombin and proconvertin. Prothrombin potency 300 to 400 times that of normal plasma per milligram of protein was repeatedly achieved. No thrombic or thromboplastic activity was detected. The lyophilized product was stable over at least 5 months. Sterile filtration of the product through a Chamberland porcelain bougie without significant loss of activity is practicable.

2. A single intravenous injection of this fraction brings the level of prothrombin to about 100 per cent when the dose calculated to do this is injected

into rabbits made deficient (< 10 per cent) in these factors by prior coumarin administration

3 The expected rise in plasma levels of prothrombin proconvertin complex and Stuart factor was also obtained following the intravenous injection of two sterile lots of this fraction in a patient deficient in these factors consequent to severe hepatocellular insufficiency These injections were tolerated without untoward incident

4 Data suggest that the half lives in the circulation after equilibrium of human prothrombin, proconvertin complex, and AHF B injected into rabbits are between 3 and 11 hours A preliminary clinical trial suggests human prothrombin, proconvertin complex, and Stuart factor when injected in man have a longer half life (50 to 55 hours)

The authors wish to thank Drs J Lewin M Steinbuch and O Prou Wartelle and Miss D Menache for their cooperation and valuable suggestions, and A J Vignal and P Petit for their technical assistance The preliminary experiments in this laboratory of Dr M J Larrieu concerning the preparation of PPB were most helpful The clinical trial of the fraction was made possible thanks to the kindness of Professor Fauvert of Hopital Beaujon Paris

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THE MORPHOLOGIC BASIS OF ANTIBODY FORMATION DEVELOPMENT DURING THE NEONATAL PERIOD

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THE rapid somatic and physiologic maturation that occurs during the latter part of gestation and during the neonatal period has provided an unusual opportunity to gain an understanding of various biologic processes[1] Our interest in the synthesis of gamma globulin and the cellular basis of antibody formation has led to a consideration of the development of these processes as revealed by changes occurring during the neonatal period Although newborn mammals have long been considered to be poor antibody producers, the extent of this defect, and its morphologic or physiologic basis, has never been precisely defined Osborn and associates[2] have presented data indicating that, during the second month of postnatal life, human infants begin to form circulating antibody However, antigenic stimulation during the first month of life results in a quantitatively lesser response than that which occurs following such stimulation at a later time during the first year A similar pattern of response has been demonstrated in various experimental animals Freund[3] has shown, using sheep red blood cells and *Salmonella typhosa* as antigens, that while an occasional rabbit will produce antibody during the second week of life, the immune response in rabbits prior to 21 days of age is quite irregular

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gamma globulin demonstrable by electrophoresis. Immunochemical determinations during pregnancy[13, 14] revealed that the actual concentration of gamma globulin remained consistently below 12 mg. per 100 ml. in spite of the slight immunologic response previously described.

During the neonatal period antigenic stimulation was provided to the child by the administration of TAB vaccine (Lederle) for immunization. One tenth millimeter was given intradermally and 0.4 ml. was given subcutaneously at the same site. This vaccine contains 1,000 million *S. typhosa*, 250 million paratyphi A, and 250 million paratyphi B organisms per milliliter. This antigen was given initially on the second day of life and then at weekly intervals thereafter during the first 2 months of life. The same quantity of antigen was then given at monthly intervals throughout the remainder of the first year of life. Cord blood was obtained from the placenta immediately after delivery and venous blood was obtained from the internal jugular vein at 2 and 7 days of age and then at weekly intervals during the first 2½ months and at monthly intervals thereafter until the baby was 14 months of age. A final specimen was obtained when the child was 2 years old. The determination of agglutinins against H, O, and B antigens was performed by serial twofold dilutions of serum in saline beginning with a 1:5 dilution. Three tenths millimeter of these dilutions was mixed with 0.3 ml. of a standardized suspension of each antigen. The sealed tubes were incubated at 37° C. and the antibody titer read the following day after standing for 2 hours at 4° C. The end point was taken as the last tube which showed agglutination. Known positive and negative serum controls were included with each series of determinations. The H, O, and B antigens used in this study were generously provided by Dr. Henry Bauer of the Minnesota State Board of Health. The baby was found to be blood group O. Isoagglutinins against heterologous blood groups were performed after blood typing using commercial typing sera (Ortho) by making serial twofold dilutions of serum in saline starting with a 1:5 dilution. Fresh cells were obtained from appropriate donors and washed 3 times in 0.145 M saline or until the supernatant was clear. When negative results were obtained in the lowest dilution, full strength serum was used to exclude the presence of minute amounts of antibody. To 0.5 ml. of each serum dilution, 0.1 ml. of a 2 per cent suspension of washed cells was added. Saline controls were run with each series of determinations. These were mixed and then incubated in a water bath at 37° C. for 30 minutes. All tubes were then centrifuged at 1,000 r.p.m. for one minute in an International centrifuge (size 2, Model V). The last tube with agglutination of the red cells was taken as the titer.

Serum gamma globulin levels have been determined by an immunochemical method,[13, 14] and by analysis of total protein using a standard biuret technique together with paper electrophoresis or free moving boundary electrophoresis. The paper electrophoretic patterns were run on a Spinco Model E electrophoresis apparatus and analyzed on a Spinco Analytrol. Free electrophoresis was performed on a Klett free electrophoresis apparatus utilizing a veronal buffer of pH 8.6 and 0.1 ionic strength. Percentage values for each protein fraction were then calculated and the gamma globulin fraction determined in reference to the total protein determination. The zinc turbidity determinations of gamma globulin were based on the method of Kunkel.[15] Lymph node biopsies were obtained from the baby in the immediate neonatal period, at 6 days of age (4 days following local antigenic stimulation), at 3 months of age, and again at 14 months of age. Imprints of the node were prepared and stained with Wright-Giemsa by the method of Downey. In addition, material from the lymph node biopsy was fixed with formol saline or absolute alcohol and stained with hematoxylin-eosin or methyl green-pyronine according to standard procedures.

RESULTS

During the neonatal period this child was clinically indistinguishable, by all morphologic and immunologic criteria, from a patient with agammaglobulinemia.[8] For example, as shown in Fig. 1, the paper and free electrophoretic

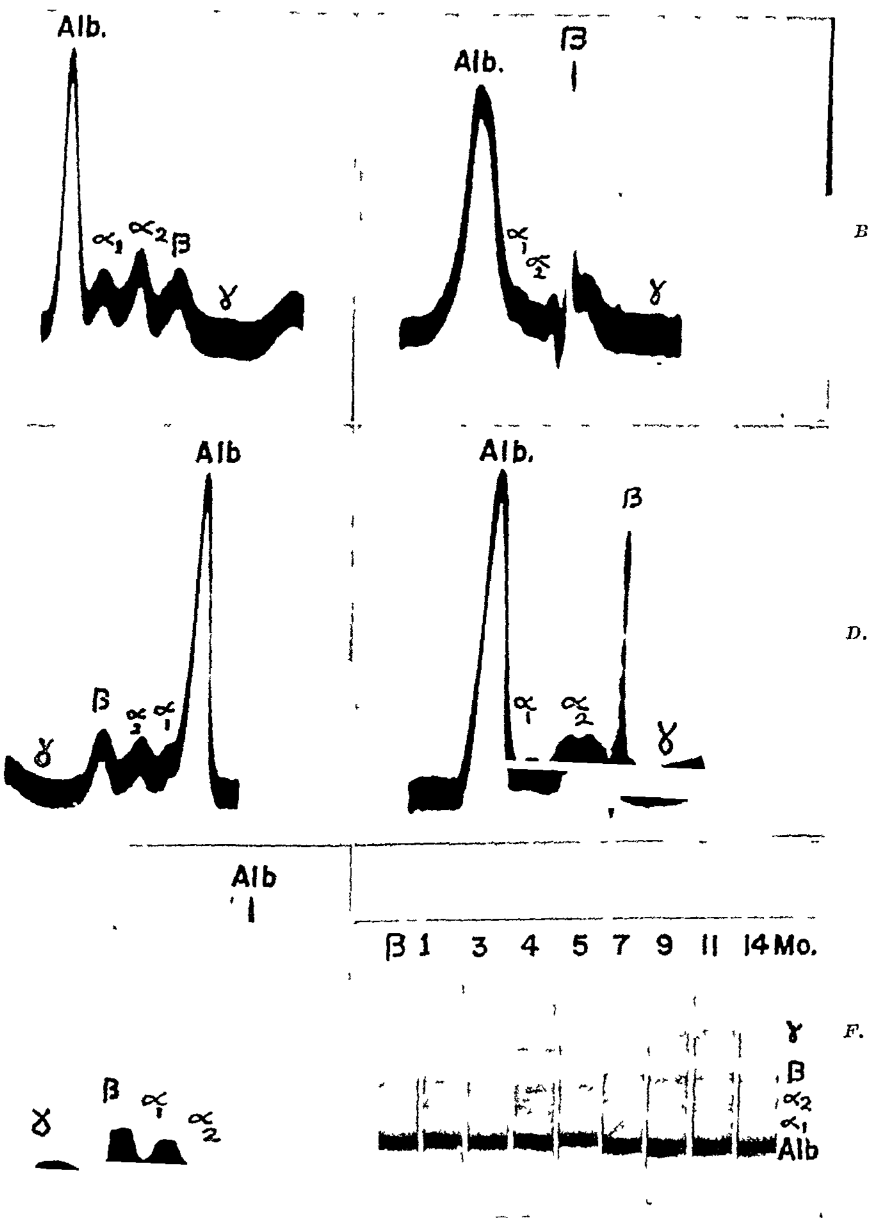

Fig 1—Electrophoretic patterns during the first year of life in normal
ıld born of an agammaglobulinemic mother. A, Ascending bound
n of the mother s serum taken at time of delivery of the child. B
maternal electrophoretic boundary of the infant's electrophoretic
ttern of serum obtained days of age Note complete absence of
mma globulin peak D, of electrophoretic pattern obtained from
by at 2 months of age. ll absent from the serum E, Ascending
undary of electrophore serum of the baby at 14 months of age.
amma globulin is now F, Serial paper electrophoretic patterns
tained on the baby mother This picture illustrates the
owth of gamma globu year of life At birth, gamma globulin
completely absent 3 months and increases rapidly and
eadily in amount during the remainder of the year of life

patterns of both the mother and baby lacked a gamma globulin peak By immunologic determination, the gamma globulin concentration in both the cord and maternal serum was 11 mg per 100 ml Plasma cells were absent from the bone marrow of both the mother and the baby (Table I) The lymph node

Table I Comparison of Numbers of Plasma Cells in Bone Marrow of Agammaglobulinemic Mother, Her Newborn Baby (an Immunologically Mature Child), and a Patient With Congenital Agammaglobulinemia

PATIENT	AGE	NO OF PLASMA CELLS PER 20,000 NUCLEATED MARROW CELLS	ANTI O AGGLUTININ TITER — MAXIMUM TITER OBTAINED*	BLOOD GROUP	ISOAGGLUTININS	
					ANTI A	ANTI B
L L Pregnant, acquired agammaglobulinemia, prior to antigenic stimulation†	33 yr	2	0	O	0	0
L L As above, 5 days following injection of antigen (third trimester)	33 yr	1	1/80*	O	0	0
R L Newborn female, born of agammaglobulinemic mother	2 days	0	0	O	0	0
R L As above, + 4 days following injection of TAB vaccine	6 days	0	0	O	0	0
R L As above, 4 days following antigenic stimulation	3 mo	23	1/160	O	1/10	0
R L As above, 4 days following antigenic stimulation	14 mo	92	1/1 280	O	1/1 280	1/320
E S Ten year old with congenital agammaglobulinemia	10 yr	0	0	O	0	0
E S As above, 4 days after antigenic stimulation	10 yr	0	0	O	0	0
T M Normal, mentally deficient child	8 yr	72	0	O	1/320	1/80
T M As above, 5 days following TAB vaccine	8 yr	138	1/640†	O	1/320	1/80

*Maximum agglutinin titer obtained 14 days following intradermal injection of 0 1 ml and subcutaneous injection of 0 4 ml of Lederle TAB vaccine containing 1 000 million heat killed *S typhosa* 250 million *S paratyphi* and 250 *S schottmulleri* organisms per milliliter

†Patient had no gamma globulin by electrophoretic method but had 11 mg per 100 ml of serum by immunochemical measurement.

tissue of the baby at the time of biopsy on the second day of life was devoid of plasma cells, and the latter did not appear in the medullary portion of the lymph node on the fourth day following antigenic stimulation During the first 2 months of life, weekly injections of typhoid paratyphoid antigens did not result in the development of a demonstrable amount of circulating antibody The serum gamma globulin concentration remained at or below 11 mg per 100 ml during the first month of the baby s life and did not show a concentration higher than this base line level until the forty second day when the concentration was found to be 115 mg. per 100 ml. Following this initial rise, the gamma globulin concentration in the baby's serum rose rapidly to levels normally observed during the first year of life. By paper electrophoresis, gamma globulin was not detectable until the sixty-seventh day of life. At this same time, on the sixty-seventh day, a titer of 1:160 against the H antigen of the TAB vaccine was first observed. At 3 months of age another lymph node biopsy was performed in the area draining the site of an intracutaneous and subcutaneous injection of typhoid-paratyphoid vaccine. The lymph node at this time possessed a morphologic appearance more like that found in adult lymph node tissue. Both primary and secondary nodules were present[*] and plasma cells were scattered throughout the medullary cords.

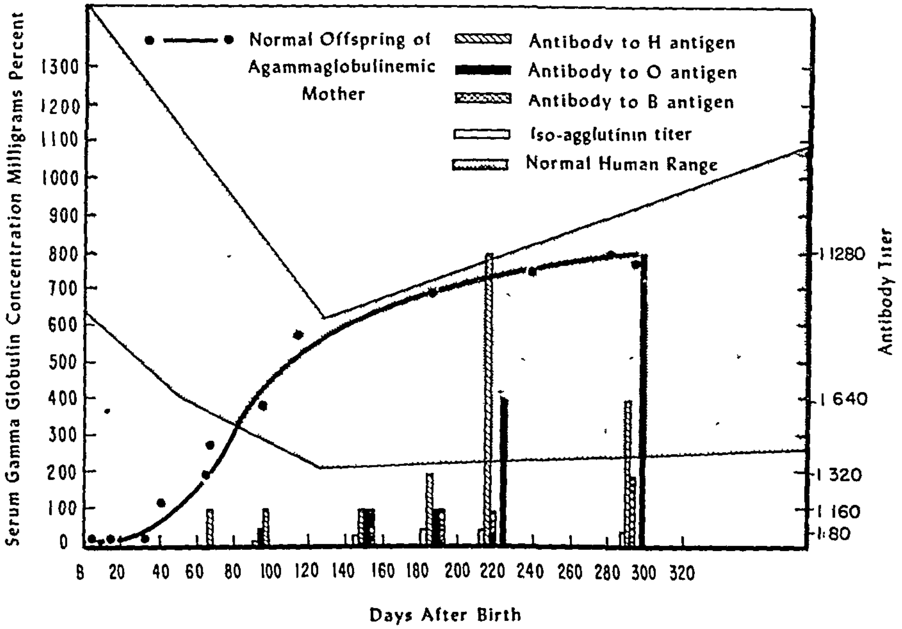

Fig. 2.—The development of gamma globulin and the formation of circulating antibody in the baby born to a woman with acquired agammaglobulinemia. The details concerning antigenic stimulation are given in the text. The shaded area over the graph indicates the normal range of gamma globulin concentration during this period of life as determined by the immunochemical technique in our laboratory. The solid line plots the gamma globulin concentration present in the baby studied while the vertical bars represent the titers of the various antibodies measured as indicated in the legends on the graph.

By 3 months of age, circulating agglutinins against the H, O, and B antigens of the TAB vaccine were present. In this baby (blood group O), isoagglutinins against both A and B cells were lacking at birth and throughout the first 2 months of life. At 3 months of age, however, isoagglutinins in a titer of 1:10 were present against A cells although the anti-B titer was still negative. By the sixth month the anti-A titer had risen to 1:80 and the anti-B titer to 1:10.

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[*] The terminology used in this paper in reference to nodular formations within the lymph nodes is defined as follows. This is modified after Gyllensten.[16] *Large primary nodule:* The diffuse collection of cortical lymphocytes bounded by the cortical trabeculae and the medullary cords. *Small primary nodule:* The dense collections of small lymphocytes found within the cortical tissue. *Secondary nodule:* Small primary that have formed centers. The center activity may be further described as early, high active, or terminal.[17]

From the third to the fourteenth month of life, antigenic stimulation with TAB vaccine was continued at monthly intervals At 14 months of age, another regional lymph node biopsy was taken 4 days following antigenic stimulation This revealed the presence of multiple small primary and scattered secondary nodules and an intense plasmacytosis within the medullary cords It may be said, then, that by 3 months of age, this child was morphologically normal and responding with antibody formation and plasma cell proliferation to antigenic stimulation By these same criteria, this child, at birth and during the first 6 weeks of life, had been indistinguishable from a child with congenital agammaglobulinemia These findings are summarized in Fig 2 where the immunologic and immunochemical observations on the baby are graphically presented In Table II the morphologic features of the baby's lymph nodes, the mother's lymph nodes and another patient with congenital agammaglobulinemia are compared to that of a normal child following antigenic stimulation In Figs 3 and 4, the morphologic observations on the lymph nodes of this child, as related to age, are presented

TABLE II MORPHOLOGIC RESPONSE OF REGIONAL LYMPH NODES TO ANTIGENIC STIMULATION*

LYMPH NODE SOURCE	LYMPH NODE SIZE	PRIMARY NODULES	SECONDARY NODULES	PLASMA CELLS
L L Agammaglobulinemic female prior to antigenic stimulation	++++	±	–	–
L L Agammaglobulinemic female 5 days following antigenic stimulation	+++++	±	–	–
R L Baby born of agammaglobulinemic mother Prior to antigenic stimulation neonatal period, 2 days of age	±	–	–	–
R L As above but 4 days after antigenic stimulation, 6 days of age	+	–	–	–
R L Four days after antigenic stimulation at 3 months of age	++	++	+	++
R L Four days after antigenic stimulation at 14 months of age	+++	++	+++	+++
E S Ten year old child with congenital agammaglobulinemia prior to antigenic stimulation	+	+		–
F S As above, 4 days after injection of antigen	++	+		–
R A Six year old female child, immunologically normal prior to antigen injection	+	+	+	+
R A As above, 4 days after first injection of antigen	++	++	++	+++
R A As above, but 4 days after second injection of antigen, secondary response	+++	+++	++++	++++

*Antigenic stimulation provided by intradermal injection of 0 1 ml and subcutaneous injection of 0 4 ml Lederle TAB immunizing vaccine as in Table I

The observations on this child, together with the data already cited from the literature and our own previous studies, have prompted the investigation that will be presented in the remaining parts of this report A brief review of the pertinent literature will be presented in each section together with the nature of the material studied and the procedures and methods utilized

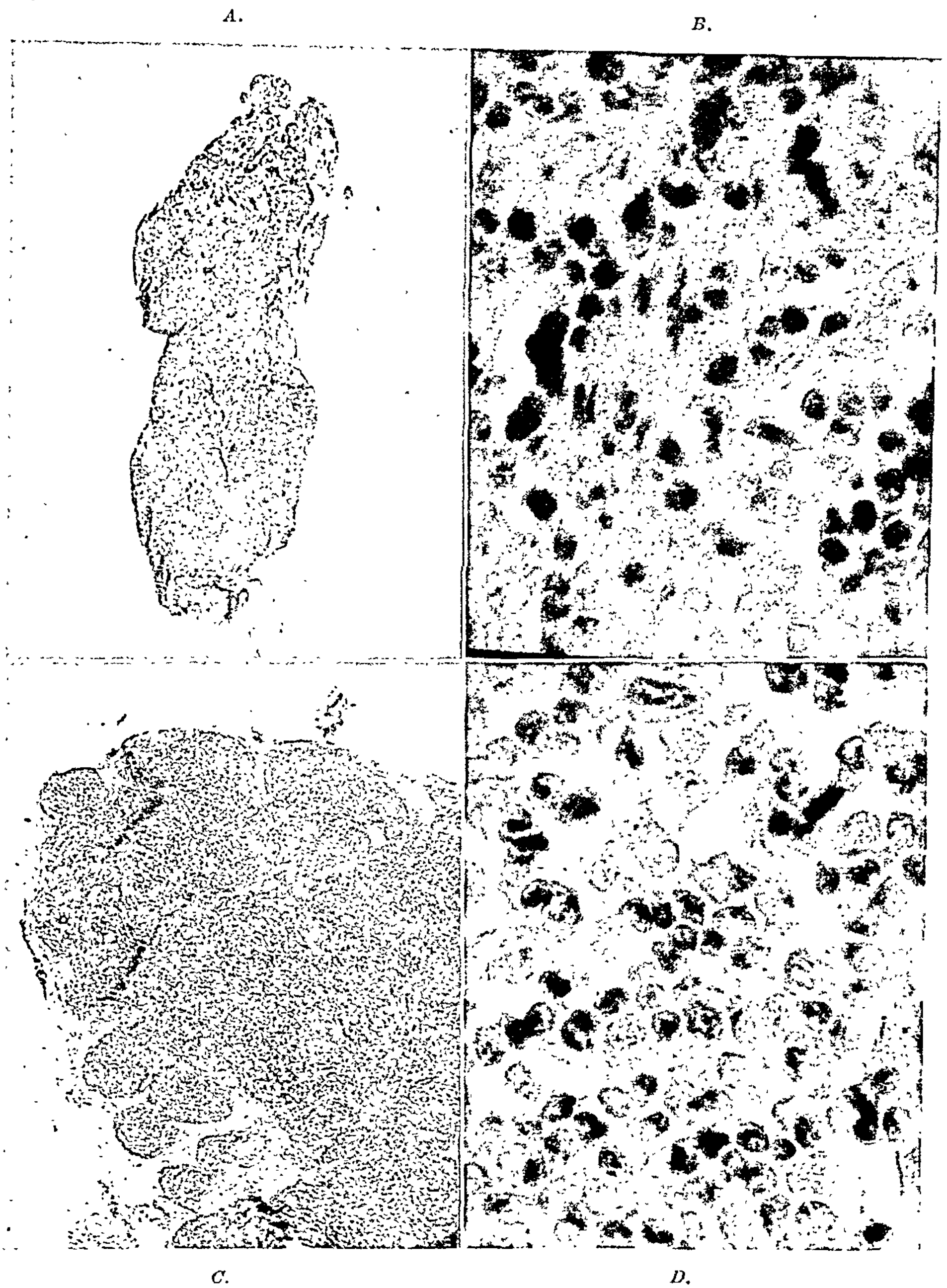

Fig. 3.—*A*, Section taken from an inguinal lymph node obtained by biopsy on the sixth y of life, 4 days after antigenic stimulation, from a child born to an agammaglobulinemic other. *Hematoxylin-eosin stain.* (Magnification ×10; reduced 1/6.) *B*, Section same as *A*. o plasma cells are present in the medullary cords. (Magnification ×800; reduced 1/6.) *C*, ection taken at 3 months of age from an inguinal lymph node of the same infant 4 days ter antigenic stimulation. Hematoxylin-eosin stain. (Magnification ×10; reduced 1/6.) *D*, ection same as *C*. A few plasma cells present in the medullary cords. (Magnification 800; reduced 1/6.)

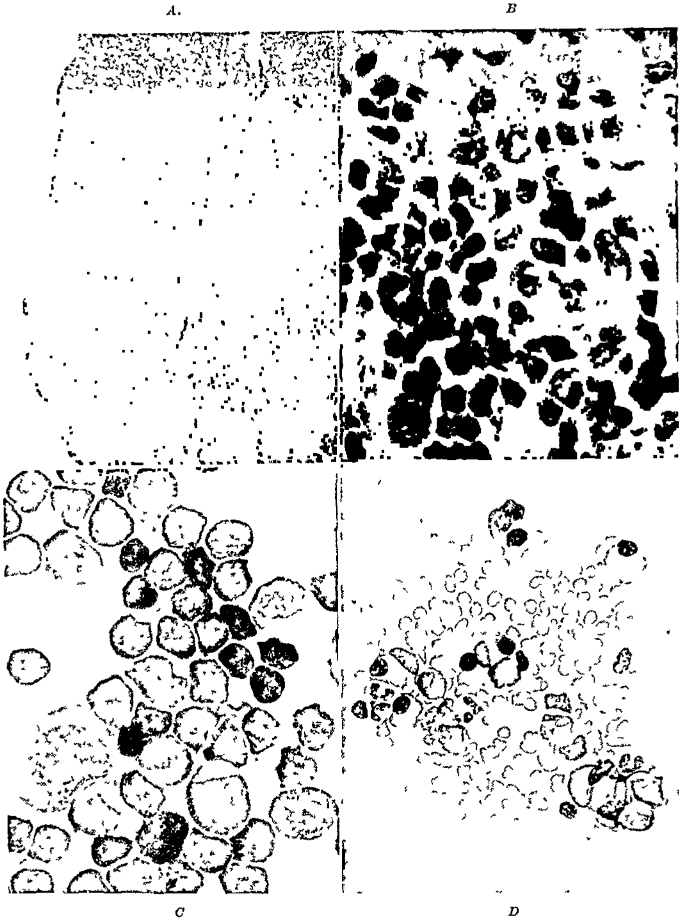

Fig. 4.—A, Section of a lymph node regional to the site of antigenic stimulation in the child born to an agammaglobulinemic mother, at 14 months of age. Hematoxylin-eosin stain. (Magnification ×10, reduced ⅙.) B, Same section as in A. Numerous plasma cells are present in the medullary cords. (Magnification ×800, reduced ⅙.) C, Imprint made from the same lymph node as A. A large blast form of the plasma cell and two more mature plasma cells are present in the field. Wright-Giemsa stain. (Magnification ×1200, reduced ⅙.) D, Direct smear of bone marrow obtained from the same child at 14 months of age. A group of 3 plasma cells is present in the field. No plasma cells were found in the marrow during the first 2 months of life. Wright Giemsa stain. (Magnification ×600, reduced ⅙.)

Part II. The Occurrence of Plasma Cells in the Bone Marrow of the Normal Newborn Infant

A number of reports are available concerning the evaluation of the bone marrow during the neonatal period.[18-21] These reports are concerned primarily with the more common cellular elements of the marrow and in none is there an attempt to estimate critically the number of plasma cells present. Raffel[22] cites a personal communication from Gormsen to the effect that plasma cells are not present in human bone marrow until the third month of life, although 2 of the 19 infants under 3 months of age examined by Sturgeon[18] were found to have 0.2 per cent plasma cells in 500 cell differential counts. In order to determine the number of plasma cells present in the normal newborn, 24 marrows were obtained from normal infants between 1 and 5 days of age. Of these, direct smears were satisfactory for making differential counts in 20 infants.

METHODS AND MATERIALS

Bone marrow was obtained by aspiration biopsy of the iliac crest under suitable aseptic technique. All marrow smears were stained by a Wright-Giemsa method. Five hundred cell differential counts were performed on these specimens. The terminology and classification of the various cells were the same as that previously used in reports from this laboratory.[23] The number of plasma cells per 5,000 nucleated cells was determined by a technique previously reported[23] that provides reproducible semiquantitative values for aspirated marrow samples.

Table III. Relative Numbers of Cells in Bone Marrow

CELL TYPES	NORMAL CHILDREN* 2.5 TO 15 YR. MEAN PER CENT	NORMAL ADULT† MEAN PER CENT	NORMAL NEWBORN‡ MEAN PER CENT	NORMAL NEWBORN‡ RANGE
Myeloblasts	1.2	2.0	1.0	0.2-2.2
Neutrophils and precursors	61.1	59.0	62.1	46.9-69.6
Eosinophils and precursors	4.7	3.5	3.8	1.6-7.5
Basophils and precursors	1.1	0.5	0.6	0.2-0.8
Lymphocytes	10.1	10.0	13.0	6.0-20.6
Plasma cells	0.4	0.4	0.0	0
Reticulum cells and monocytes	1.7	2.2	3.9	1.8-6.4
Megakaryocytes	0.4	0.4	0.3	0.0-0.6
Erythroid cells	19.2	22.0	14.7	7.6-26.4

*Thirteen cases, Good and Campbell: Am. J. Med. 9: 330, 1950.
†Wintrobe: Clinical Hematology, Philadelphia, 1946, Lea & Febiger.
‡Twenty cases.

RESULTS

From the data presented in Table III it will be seen that, with respect to the more common elements of the marrow, there are no striking differences between those newborn infants and older normal children and adults. It should also be noted, however, that no plasma cells were encountered in the marrows of these newborn infants whereas these cells are regularly present in the marrow from persons of older age groups.

In order to arrive at a more quantitative definition of the number of plasma cells present in the newborn child, 5,000 nucleated cell counts were performed on the same marrow specimen When the number of marrow plasma cells is estimated in this fashion, there is at once a striking difference evident between the newborn and older age groups In Table IV the results obtained from newborn marrows are compared with the results in older children and adults using the same method In none of the 5,000 cell counts performed on these newborn infants were any plasma cells encountered This state, then, is comparable with that observed in children suffering from congenital agammaglobulinemia where plasma cells are virtually absent from the marrow [24]

TABLE IV BONE MARROW PLASMA CELLS

GROUP	AGE	PLASMA CELLS PER 5,000 NUCLEATED MARROW CELLS	
		MEAN	RANGE
Normal children 12 cases	2 5 to 15 yr	19 8	11 32
Normal adults 15 cases	18 to 41 yr	31 0	8 61
Normal newborn 20 cases	1 to 5 days	0 0	0

PART III THE DEVELOPMENT OF PLASMA CELLS IN THE LAMINA PROPRIA OF THE HUMAN INTESTINE

Of the various areas of the body where lymphoid tissue is present, there is no area that contains large numbers of plasma cells as regularly as the intestine Davis[25] has reported the development of plasma cells in tonsillar tissue during the first 3 months of life He noted that they were not present before the second or third week of life but had invariably developed by the eighth week of life in all of the 17 specimens from children under 3 months of age He also mentioned that plasma cells appeared in the lamina propria of the appendix shortly after birth The studies to be reported in this part of the paper will clearly relate the development of plasma cells in the lamina propria of the ileum and appendix to age

METHODS AND MATERIALS

Specimens were collected from a series of consecutive postmortem examinations done on children at our hospital Many of the specimens that were obtained were taken from children dying of *congenital heart disease or from some other congenital malformation* incompatible with life A few of the children in this series died of neoplastic disease, and a few more were from children dying of infection At each post mortem two specimens were taken One was obtained from the ileum and the other from the appendix Half of each specimen was fixed in 10 per cent formol saline and the other half fixed in absolute alcohol These were subsequently stained by hematoxylin eosin and with methyl green pyronine respectively On microscopic examination, it was found easiest to arrive at a semiquantitative grading of the number of plasma cells present from the material stained with methyl green pyronine Although all gradings are from material stained by that method, no essential disagreement was observed when the plasma cell concentration was evaluated using either method of fixation or staining These specimens were graded on a rough quantitative scale from 0 to 4+ The specimens were graded blindly by one observer without knowledge of the age or the disease of the patient from whom the material was collected The exact age and cause of death were later taken from the hospital records

RESULTS

Graphs recording the degree of plasmacytosis observed in the lamina propria of the appendix and ileum are plotted against the age of the patient in Figs. 5 and 6. There is a striking absence of plasma cells in the newborn intestine. This area in the newborn consists of a loose meshwork of reticular tissue. Lymphoid tissue, in general, was sparse and poorly developed when compared with that present in older children. In the 14 appendices and the fifteen specimens of ileum examined in children less than one month of age, there was

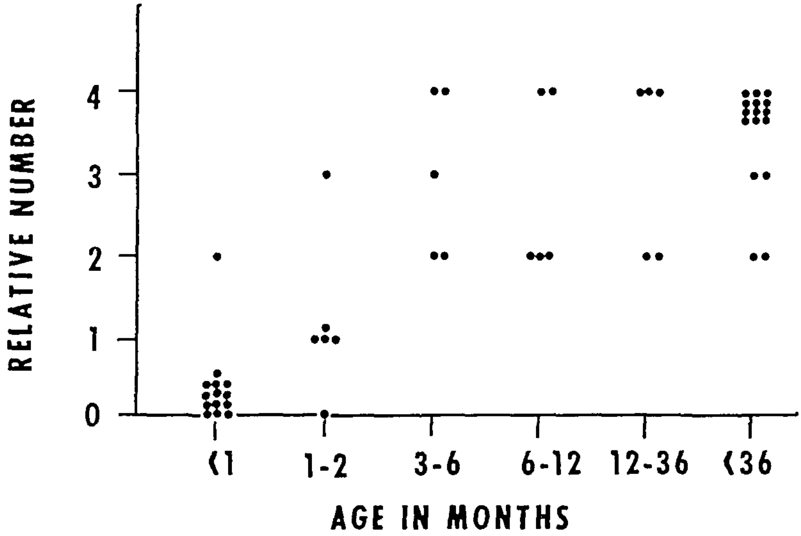

Fig. 5.—The relative number of plasma cells occurring in the lamina propria of the appendix is plotted against groups of children of increasing age.

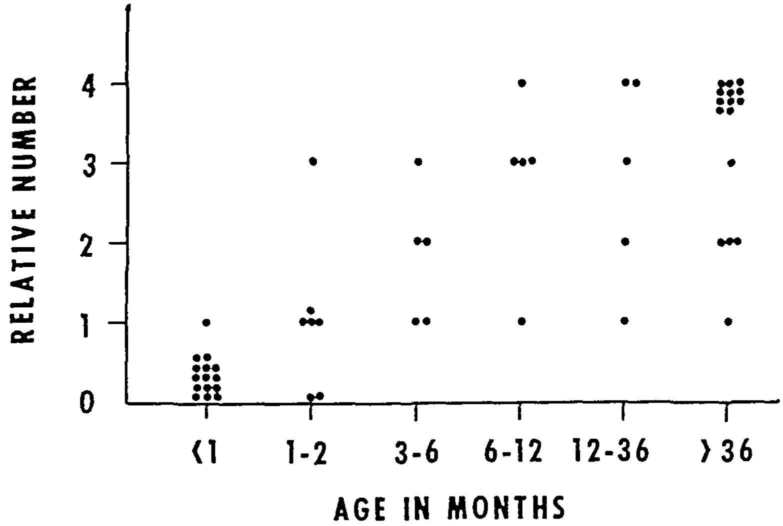

Fig. 6.—The relative number of plasma cells occurring in the lamina propria of the ileum is plotted against groups of children of increasing age.

only one exception to this uniform absence of plasma cells This occurred in a child who died at 3 weeks of age This child possessed plasma cells in the appendix in numbers sufficient to result in a grading of 2+ while the ileum was graded as 1+ In the material examined, plasma cells were invariably present by the age of 6 months, and by 3 years of age most of the children had a level of plasma cells that was graded as 4+ Between 1 and 6 months of age a transitional period was observed and plasma cells ranged from 0 to 4+ The appearance of plasma cells is associated with a gradual increase in the total amount of lymphoid tissue in the gut Secondary nodules were first found in an infant of 3 weeks of age and were almost invariably present in the specimens from older children The observations comparing the plasma cell and lymphoid tissue deficit in the intestinal tissue of the neonate, their gradual appearance during the first few months of life, and their abundance in an older child are illustrated in Fig 7

Part IV The Occurrence of Plasma Cells Following Antigenic Stimulation During the Neonatal Period in the Rabbit

A number of investigations of the lymphoid tissues present during the newborn period have been made in a variety of animal species These have been concerned primarily with the grosser elements present and with the general structure of the lymph nodes as related to age Gyllensten[16] in a study of the guinea pig noted that the lymph nodes seemed relatively mature at birth Secondary nodules were found to develop between the first and twenty third days of life He noted that plasma cells were present in the newborn guinea pig and that they did not change appreciably during the first 35 days of life* In a subsequent study, he showed that antigenic stimulation by infection with *B pyocyaneus* did not induce secondary nodule formation prior to the time of the normal appearance of these centers Denz[26] has examined human lymph nodes from individuals of various ages No mention was made of the cellular components present, although he does describe the various structural components as they vary with age and with the area of the body concerned The morphologic response to antigenic stimulation has been studied by a number of authors using a great variety of antigens In general, they have shown that, in addition to the usual lymphoid hyperplasia that results late in the course of antigenic stimulation, there is a specific plasmacellular response occurring just prior to the appearance of circulating antibody in the blood The rise in antibody content of the lymph occurs between the peak plasmacellular response and the occurrence of circulating antibody in the blood[27] The plasma cells developing in response to antigenic stimulation have been shown to contain antibody to the antigen concerned[28, 29]

METHODS AND MATERIALS

Rabbits were identified at birth in order to insure the accuracy of the ages given They were kept with the mother during approximately the first 6 weeks of life in nest boxes that were maintained in separate cages They were weaned at about 6 weeks of age, following which they were fed a diet of Purina rabbit pellets. The breeding stock used was the New Zealand White Rabbit. At the appropriate age, approximately two-thirds of the rabbits in each litter were given injections into each hind foot pad with 0.05 ml. of the antigenic material being studied. The injected rabbits were marked by tattooing the ear with India ink. Antigenic stimulation was provided by either a saline solution of crystalline bovine serum albumin (BSA)* given in a dosage of 20 mg. per

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*We have by the imprint and stained section techniques observed both this relative maturity of guinea pig lymph nodes and the presence of plasma cells

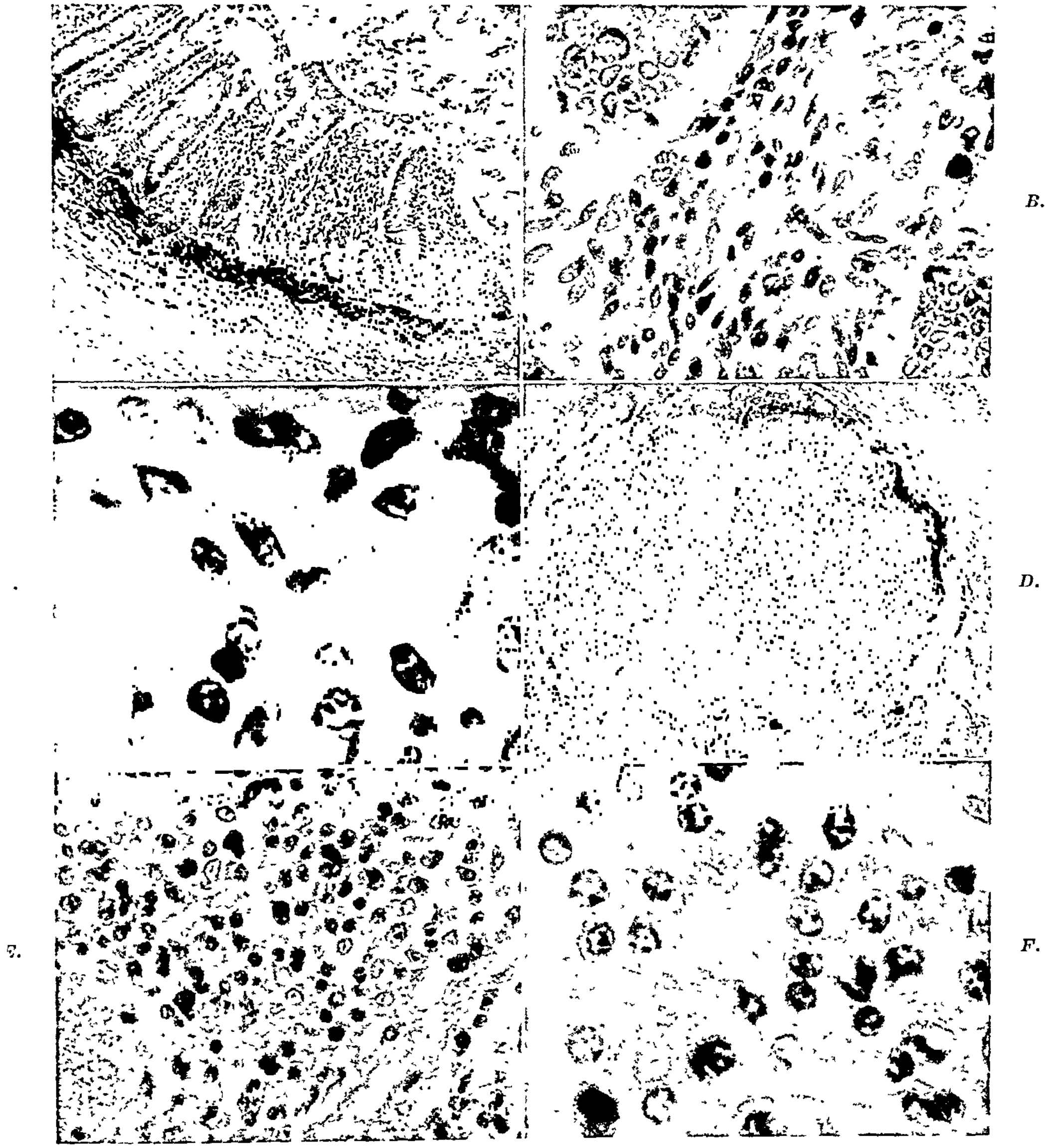

Fig. 7.—*A*, Section from the appendix of a child one day of age. Only a sparse amount of lymphoid tissue is present. Methyl green-pyronine stain. (Magnification ×100; reduced ⅙.) *B*, Same section as *A*. (Magnification ×300; reduced ⅙.) *C*, Same section as *A*. Plasma cells are absent from the lamina propria. [illegible] ×800; reduced ⅙.) *D*, Section from the appendix of a child 6 years of age. [illegible] is present. (Magnification ×25; reduced ⅙.) *E*, Same section as *D*. [illegible]; reduced ⅙.) *F*, The same section as *D*. Plasma cells are abundant in the lamina propria. (Magnification ×800; reduced ⅙.)

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*Obtained from Armour & Company, Chicago, Ill.

kilogram or the same amount of BSA emulsified in Freund's complete adjuvant (Difco) BSA was dissolved in cold 0 145 M sterile pyrogen free saline to give a concentration such that the total dosage of 20 mg per kilogram would be contained in 0 1 ml When the BSA was combined with Freund's Adjuvant, the appropriate amount was dissolved in one quarter of this amount of pyrogen free saline At the time of injection this solution was emulsified in the adjuvant mixture in a ratio of 1 3 The dosage of BSA and the total volume of the injection were thus kept constant for each group The remaining animals in the litter were kept for morphologic controls Half of the animals injected, together with the controls, were sacrificed 5 days following antigenic stimulation and the popliteal lymph nodes removed from each side for morphologic investigation The lymph nodes were divided in half, longitudinally, so that the cut passed through the hilum Half of the node was used for making imprints The other half of the node was placed in either 10 per cent formol saline for subsequent staining by hematoxylin and eosin or in absolute alcohol and stained with methyl green pyronine This expedient of bilateral antigenic stimulation and the division of material for sectioning was necessary because of the extremely small size of the lymph nodes in the very young rabbits It was thus possible to obtain, from each animal lymph node imprints from each side and section material stained with hematoxylin eosin from one side and material stained with methyl green pyronine from the other side The terminology used in describing the various cellular components is that of Sundberg[30] The number of plasma cells present was again graded on a scale ranging from 0 to 4+ The remaining half of the animals injected in each litter were exsanguinated 12 days following injection of the antigen The blood was allowed to clot at room temperature for one hour and was then separated after standing overnight at 4° C The serum was frozen at -70° C and stored at -25° C until antibody determinations were performed On these sera the antigen antibody equivalence point was estimated from a preliminary determination as outlined by Kabat and Meyer[13] Standard solutions of BSA (Armour) were prepared by dissolving appropriate amounts in nitrogen free 0 145 M NaCl and their total nitrogen content determined by the Markham modification of the micro Kjeldahl procedure Volumetric dilutions of these standard solutions were prepared so that 4 concentrations would be obtained extending from approximately 1 0 to 6 μg of nitrogen per milliliter Then, 0 5 ml of the serum to be tested was added to 0 5 ml of each of the 4 antigen solutions These tubes were then incubated for one hour at 37° C and subsequently stored for 5 to 7 days at 4° C The samples were then centrifuged for one hour at 4° C in an International centrifuge Model PR 1 The supernatant was then carefully decanted and used for a capillary tube analysis to demonstrate the presence of antigen The immune serum used for the capillary tube precipitin determinations was obtained from a rabbit hyperimmunized to BSA by the repeated intravenous administration of this antigen in saline solution Using this technique, concentrations of BSA as low as 0 5 μg of nitrogen per milliliter are detectable Thus when free antigen is detectable in the supernatant from the mixture of serum with an equal volume of the antigen solution, at a concentration of 1 0 μg of nitrogen per milliliter, virtually none of the original antigen could have been precipitated by antibody The value for the maximum amount of antigen bound was then used to calculate the approximate antibody content A value of 5 0 for R (combining ratio) was assumed in all calculations Figs 8 and 9 describe graphically the experimental plan by which the relationship between the morphologic response and the production of antibody was worked out in these studies In the part of the study summarized in Fig 8, saline solutions of BSA were injected intradermally in each foot pad at birth and at 7, 14, 28, and 56 days of age The rabbits in each litter to be studied for their morphologic response were sacrificed 5 days after antigenic stimulation The members of each litter that were to serve as morphologic controls were also sacrificed at this time Antibody determinations were performed on the serum obtained from littermates on the twelfth day following antigenic stimulation In the portion of the study illustrated in Fig 9, a serial study was performed of the morphologic and antibody responses of animals injected either at birth, 7, or 28 days of age with BSA emulsified in Freund's complete adjuvant. At the times of sacrifice, indicated on the graph, the popliteal nodes were removed and blood was obtained for antibody determinations. Normal lymph nodes were also obtained from littermates of these animals at various ages.

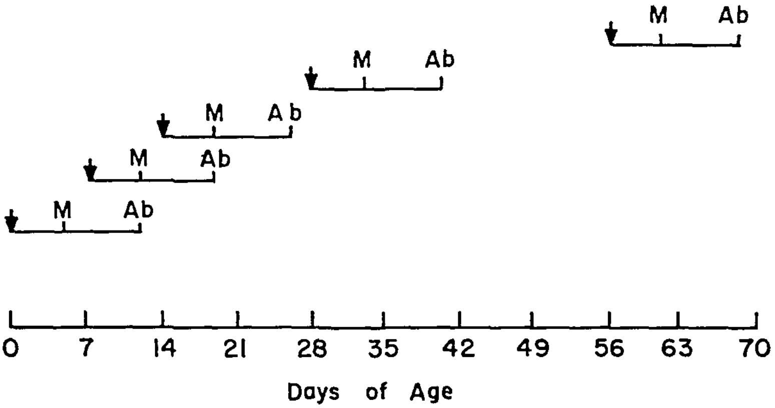

Fig. 8.—Response to antigenic stimulation with BSA in saline solution as a function of age. Outline of the experimental groups chosen to demonstrate the relationship of age to the morphologic and antibody responses in rabbits of different ages. The arrows denote the time of stimulation, M indicates the time of sacrifice for morphologic observation, and Ab denotes the time at which bleedings were taken for antibody determinations.

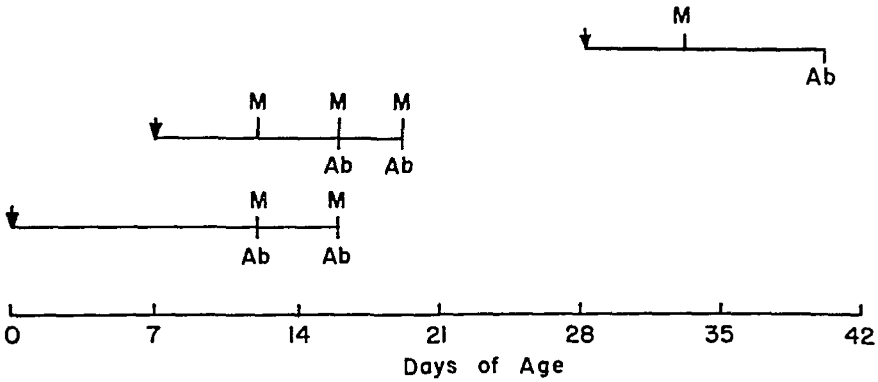

Fig. 9.—Serial study of response to antigenic stimulation with BSA in adjuvants. Outline of the experimental groups chosen to demonstrate the response to intensive antigenic stimulation in immature rabbits. The arrows denote the time of stimulation, M indicates the time of sacrifice for morphologic observation, and Ab denotes the time at which bleedings were taken for antibody determinations.

RESULTS

Morphologic Descriptions of the Control Lymph Nodes From Rabbits at Various Ages.—

Five days of age: The unstimulated rabbit lymph nodes at this age were very small, averaging 1 to 2 mm. in diameter. The sectioned material revealed a poorly organized lymphoid tissue with a thin rim of poorly defined cortex and a thin meshwork of reticular tissue in the medullary zone. Only very infrequent small primary nodules were present. In the imprinted and sectioned material a great variety of cellular elements could be identified. Scattered elements indicating active erythropoiesis were seen. These cells were mainly polychromatophilic normoblasts, although at times even more immature erythroid elements were found. Granulocytopoiesis was not prominent, but rare myelocytes and promyelocytes were present. Both heterophils and eosinophils were identified, and an extremely rare megakaryocyte was encountered. Mast cells were prominent in the imprinted material. All elements of lymphocytopoiesis were present and, as noted by Downey,[31] occasional forms indistinguishable from myeloblasts were observed. No plasma cells were found in any of the material examined. Fig. 10 illustrates some of the various morphologic changes occurring as a function of age in normal rabbits.

Twelve days of age. The popliteal lymph nodes at this age are still approximately the same size as those in the 5-day-old rabbit. The cortex, however, is better defined and more sharply delineated from the medullary portion. Occasional small primary nodules, but no secondary nodules, were present. The medullary cords remained immature and consisted of a loose meshwork of reticular cells. Qualitatively, the same variety of cellular elements was found, although there was a definite reduction in erythropoiesis and myelopoiesis. Occasional rare immature granulocytes were still present, however. Mast cells were less numerous than in the lymph nodes of 5-day-old rabbits. Plasma cells were again absent.

Sixteen days of age. These lymph nodes were larger than the 12-day nodes (2 to 4 mm.). The medullary cords were better defined and occasional rare secondary nodules were present in the cortex. No plasma cells could be found in any of the lymph nodes examined.

Nineteen days of age. Although still quite small, the lymph nodes at this age were well formed. The cortical area was well delineated, and both primary and secondary nodules were present. The medullary cords, although still immature, showed a higher degree of organization. Cellular elements other than those of lymphocytopoiesis were uncommon. More eosinophils were present than were observed in the nodes of the younger rabbits. In 4 of the 5 rabbits examined at this age, plasma cells were completely absent. In the one exception, plasma cells were present although extremely rare.

Thirty-three days of age. The general structure of the node at this age is similar to that in the 19-day-old rabbit. Rare heterophil and eosinophil myelocytes were present. Mature heterophils and eosinophils were not uncommon. No evidence of erythropoiesis was found. Rare plasma cells were found in the lymph nodes from 2 of the 4 rabbits examined.

Sixty-one days of age. By this age the lymph nodes examined were considerably larger than those of the previous groups. They were still slightly smaller than those of normal adult rabbits. Plasma cells were scattered throughout the medullary cords and both primary and secondary nodules were present

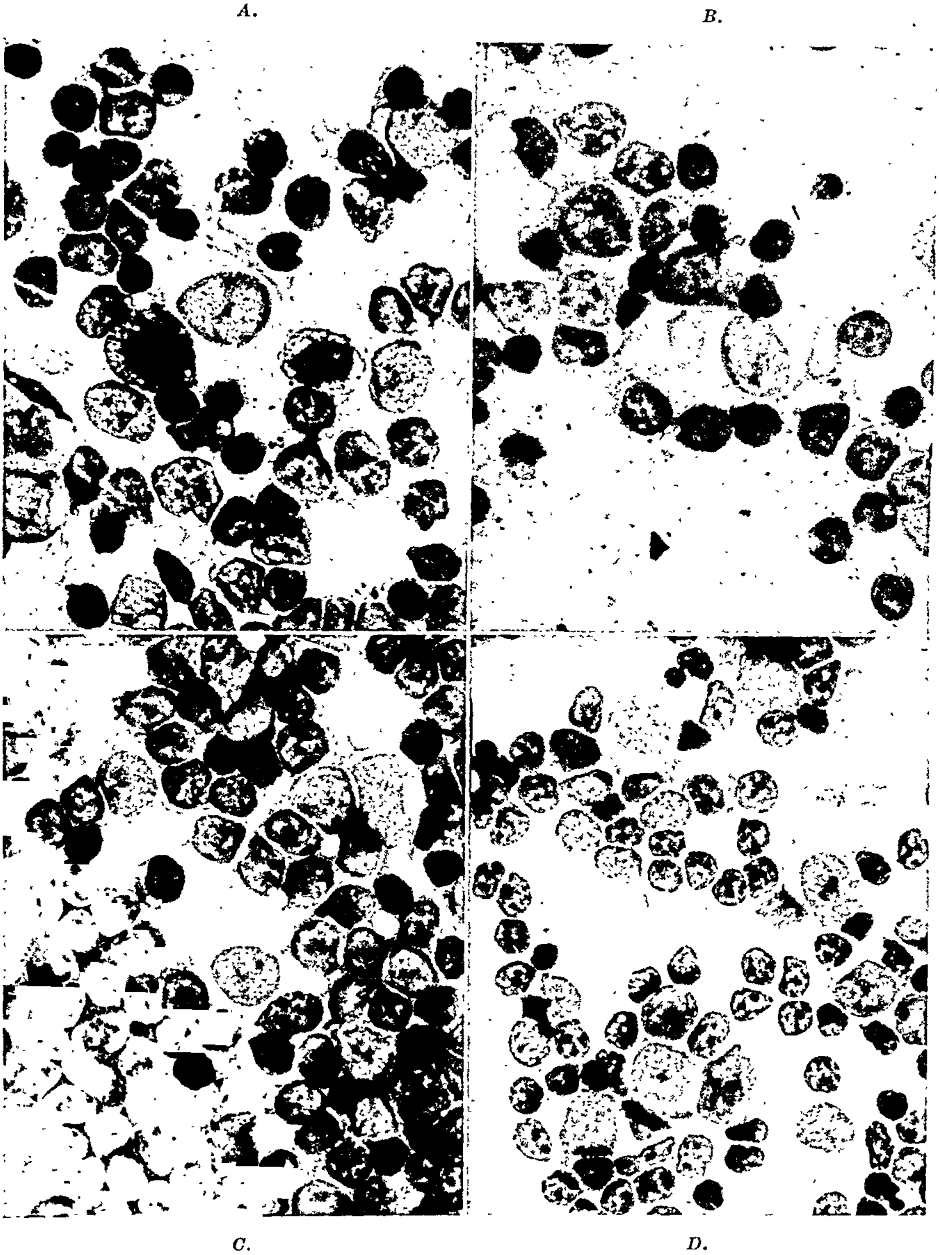

Fig. 10.—*A*, Imprint made from the popliteal lymph node of a 5-day-old normal rabbit. Wright-Giemsa stain. Note the presence of mast cells. Note mast cells and lymphocytes *in various stages of maturation. An activated reticular cell is in the center of the field.* In one corner is a [illegible] normoblast. No plasma cells could be found. (Magnification ×1,200; [illegible] imprint made from lymph node of 12-day-old normal rabbit. Wright-Giemsa [illegible] lymphocytes *in various stages of maturation and a macrophage* in the center of the field. No plasma cells present. A normoblast is present in one corner of the field. (Magnification ×1,200; reduced ⅕.) *C*, Imprint made from 33-day-old normal rabbit. Wright-Giemsa stain. The various stages of *lymphocytopoiesis* are again illustrated. No plasma cells could be found in the material examined in this rabbit. (Magnification ×1,200; reduced ⅕.) *D*, Imprint made from 61-day-old normal rabbit. This field, though showing a concentration of plasma cellular elements rarely found in normal rabbits at this age, illustrates all the various stages that may be found in very small numbers. (Magnification ×1,000; reduced ⅕.)

Adult rabbits (2 0 Kg) These were quite similar to the 61 day old lymph nodes previously described Plasma cells were more numerous, however, and scattered small nests of these cells could be found in the medullary cords

TABLE V PLASMACELLULAR AND ANTIBODY RESPONSE TO INTRADERMAL STIMULATION WITH BSA IN SALINE SOLUTION

GROUP	AGE WHEN STIMULATED					
	< 24 HR	7 DAYS	14 DAYS	28 DAYS	56 DAYS	ADULT
Grading of plasma cells present in lymph node*						
A Control	None	None	None	None	+	+
	None	None	None	None	+	++
	None	None	None	Rare	Rare	+++
		None	None	Rare	++++	+
		None	Rare			+++
B BSA 20 mg /Kg	None	None	None	++	++	++++
	None	None	None	++	++	+++
	None	None	None	++	+++	+++
	None	None	None	++++	++	+++
	None	None	Rare	+++		++++
Antibody response†	0/3	0/5	0/5	4/5 (0 15)	3/4 (0 15)	

*All lymph nodes removed for examination 5 days after antigenic stimulation

†Determined from bleedings made on littermates 12 days after antigenic stimulation The numerator gives the number of animals showing a detectable response and the denominator the number stimulated The range of response is in parentheses

Morphologic and Antibody Response to a Saline Solution of BSA at Various Ages—Intradermal antigenic stimulation in animals younger than 28 days did not result in either a plasmacellular or an antibody response Morphologic examinations were performed in 15 rabbits younger than 28 days Table V presents the comparative morphologic data obtained in these rabbits at the various ages The first group to show a plasmacellular response were those rabbits that were 28 days of age at the time of antigenic stimulation When the regional lymph nodes were removed 5 days later, a very striking increase in the number of plasma cells had occurred Table V also presents the results of the antibody determinations performed on bleedings taken from littermates on the twelfth day after antigenic stimulation Antibody determinations were performed in 13 rabbits younger than 28 days at the time of stimulation None of these rabbits had detectable amounts of antibody at this time The first animals that produced antibody in response to the injection of a saline solution of BSA were those that had been 28 days old at the time of stimulation Four out of the 5 animals in this age group had produced antibody by the time they were bled 12 days after antigenic stimulation

In rabbits older than 28 days at the time of antigenic stimulation, both a morphologic and antibody response occurred regularly The natural occurrence of plasma cells in the popliteal nodes of older rabbits made the morphologic response to primary antigenic stimulation less obvious than in younger animals The difference between the morphologic response in immature and mature rabbits is illustrated in Fig 11

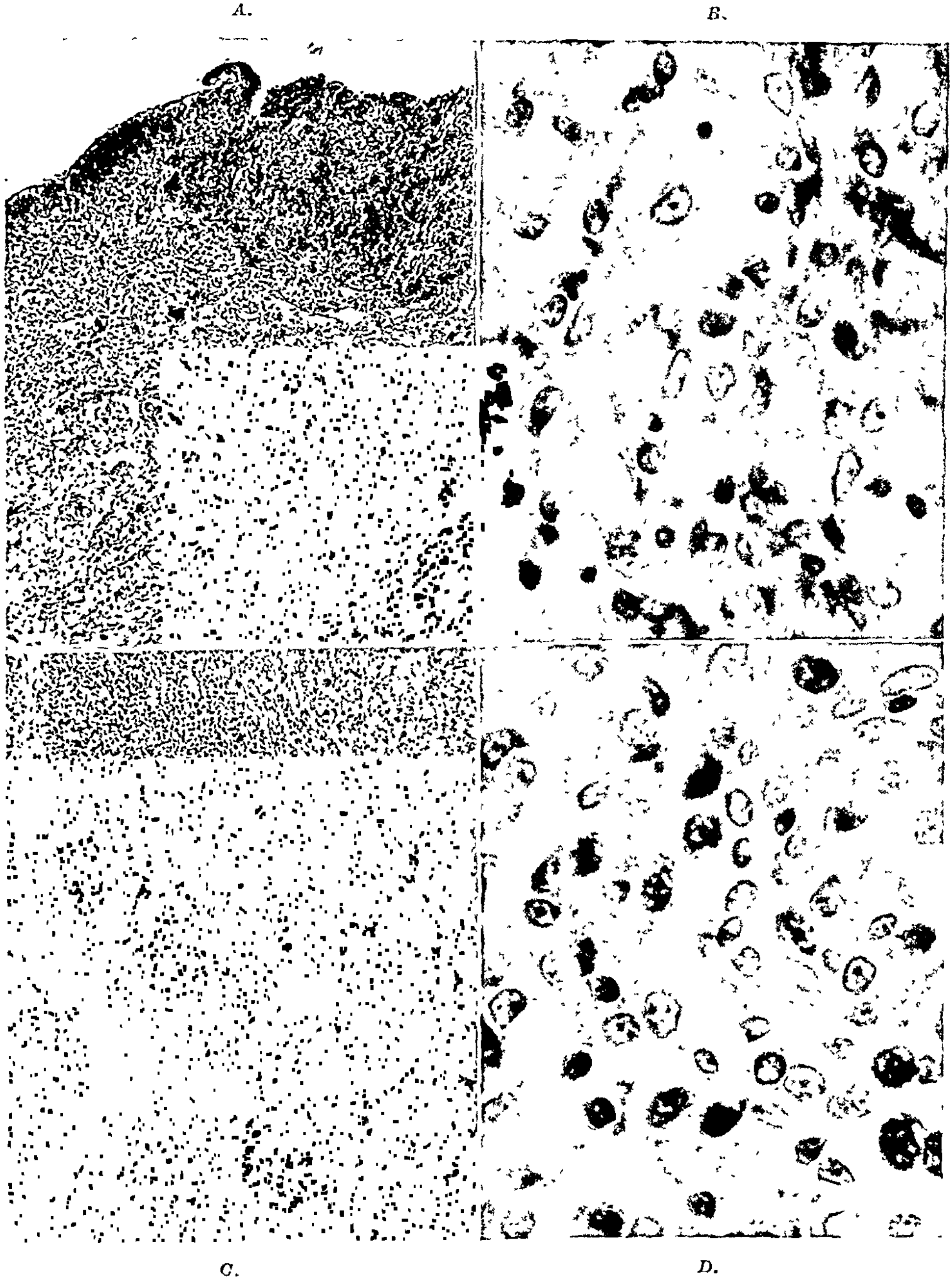

Fig. 11.—*A*, Section taken from the popliteal lymph node of a 12-day-old rabbit 5 days after local intradermal stimulation with a saline solution of BSA. Stained with methyl green-pyronine. The cortical area is somewhat thicker than in younger animals, but contains only scattered small primary nodules. No secondary nodules were present. (Magnification ×100; reduced $\frac{1}{6}$.) *B*, Same lymph node as *A*. Plasma cells are absent from the medullary cords. (Magnification ×800; reduced $\frac{1}{6}$.) *C*, Section taken from regional node of 61-day-old rabbit 5 days following antigenic stimulation with a saline solution of BSA. Both small primary and secondary nodules are present in the relatively thick cortical area. The medullary cords are clearly defined. (Magnification ×100; reduced $\frac{1}{6}$.) *D*, Same lymph node as *C*. Plasma cells are present in the medullary cords. (Magnification ×800; reduced $\frac{1}{6}$.)

The Morphologic and Antibody Responses to BSA Emulsified in Freund's Complete Adjuvant at Various Ages—In order to extend the observations made with a saline solution of a purified protein antigen, a highly potent antigenic mixture was chosen to define more closely the age at which an immunologic response first became evident To provide an intensive antigenic stimulus, BSA was incorporated in a water in oil emulsion containing *Mycobacterium butyricum*[3] In this series of investigations, antigenic stimulation was provided by this antigenic mixture at birth and at 7 and 56 days of age Except for this last group of older rabbits, the time intervals chosen in preceding series of investigations (Fig 8) were not satisfactory for a more critical investigation of the age at which an immunologic response was first demonstrable For this reason those animals that had been stimulated either at birth or at 7 days of age were sacrificed serially at frequent intervals and both antibody determinations and the morphologic response were evaluated in the same animal (Fig 9) These results are summarized in Table VI Prior to the nineteenth day of life, even after this intensive antigenic stimulation, antibody did not appear in the circulation In those rabbits injected at 7 days of age, only occasional plasma cell precursors were present in the medullary cords on the twelfth day of life, but by the sixteenth day an intense proliferation of immature plasma cells was

TABLE VI PLASMACELLULAR AND ANTIBODY RESPONSE TO INTRADERMAL STIMULATION WITH BSA INCORPORATED IN ADJUVANTS

AGE AT TIME OF SACRIFICE	AGE WHEN STIMULATED						PLASMA CELLS IN CONTROL LYMPH NODES
	DAY OF BIRTH		7 DAYS OLD		28 DAYS OLD		
	CELLULAR RESPONSE	ANTIBODY RESPONSE	CELLULAR RESPONSE	ANTIBODY RESPONSE	CELLULAR RESPONSE	ANTIBODY RESPONSE	
12 Days	Rare*		Rare				None
	Rare		Rare				None
	Rare	0/5†	Rare				None
	Rare		Rare				None
	Rare		Rare				None
16 Days	Rare		++++				None
	Rare	0/3	++++				None
	Rare		++++	0/5			None
			++++				None
			++++				None
19 Days			++++				None
			++++	8/8			None
			++++	(8 17)			None
							None
							Rare
33 Days					+++		None
					++++		None
					++++		Rare
					++++		Rare
					++++		
40 Days						5/5 (All > 27)	

*Grading of plasma cells present in regional lymph node at the time of sacrifice

†Numerator gives number of animals having detectable antibody in bleeding taken at time of morphologic observation Denominator gives number of animals tested

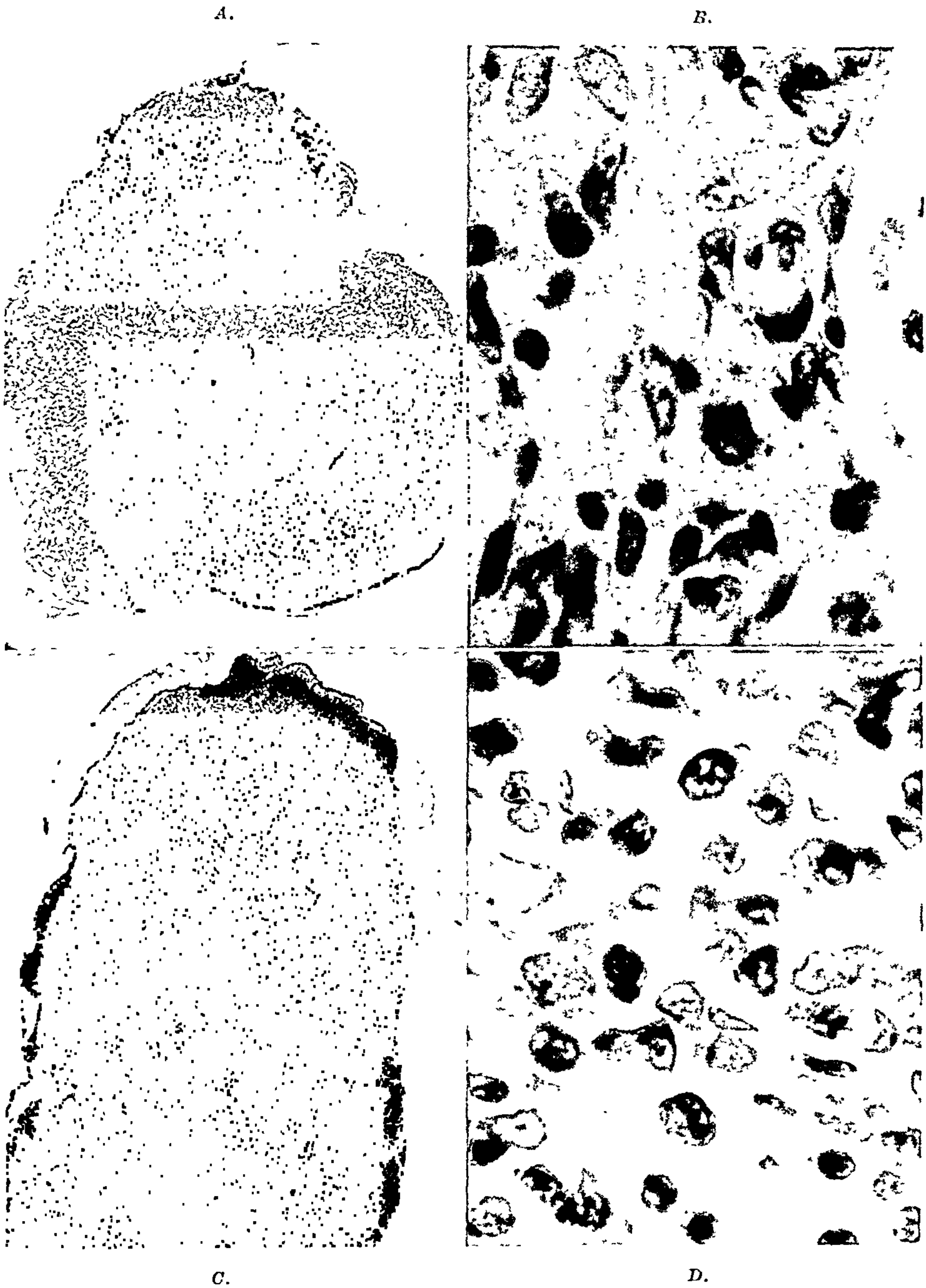

Fig. 12.—*A*, Section taken from the popliteal lymph node of a 12-day-old rabbit. BSA incorporated in Freund's adjuvant mixture was given on the day of birth. Stained with methyl green-pyronine. The lymph node is greatly enlarged for a rabbit of this age though the gross structure is much the same. (Magnification ×10; reduced ⅙.) *B*, Same section as *A*. No plasma cells are evident in the medullary cords. (Magnification ×800; reduced ⅙.) *C*, Section taken from the popliteal lymph node of a rabbit 19 days old. BSA incorporated in Freund's adjuvant mixture was given 12 days before sacrifice on the seventh day of life. The gross [illegible] to *A* although this lymph node is even larger. Methyl green-pyronine [illegible] ×8; reduced ⅙.) *D*, Same section as *C*. Plasma cells are present. [illegible] reduced ⅙.)

evident in the regional node Injections performed at the time of birth did not accelerate this process Prior to the sixteenth day of life, in those animals injected at birth, there was neither a detectable morphologic response nor antibody production

Although a plasmacellular response did not develop before the sixteenth day of life, there were a number of striking morphologic changes that occurred in these immature rabbits These lymph nodes increased greatly in size within the first 5 days after stimulation It was determined from the sections and imprints that this increase in size was due to an extensive proliferation of the reticulum with a great increase in the thickness of the cortex Reticular cells and hematopoietic reticular cells, together with macrophages and epithelioid cells, were striking in their numbers In older rabbits stimulated with such adjuvant mixtures, these changes occurred together with an extensive proliferation of lymphocytes, a formation of secondary nodules and a proliferation of plasma cells These later changes were regularly observed as early as 5 days after stimulation in more mature animals It appears that, in order for lymph nodes to manifest the morphologic changes which regularly occur after antigenic challenge in older animals, some form of physiologic maturation must ensue A comparison is made in Fig 12 of the popliteal lymph nodes from a 12 day old rabbit injected into the foot pad on the day of birth with BSA in adjuvants and from a 19 day old rabbit injected at 7 days of age

DISCUSSION

Together with the assumption of an extrauterine existence, there occurs the necessity to develop those immunologic functions which are characteristic of adult animals While in utero the developing fetus is protected by the defenses of the mother and the barrier provided by the placenta, but from the time of birth he must defend himself against the potential pathogens of the environment During the neonatal period, however, both human beings and rabbits are deficient in their immunologic capacities, and it is at this particular time that the neonate is peculiarly susceptible to a variety of severe and fulminant bacterial infections which, during childhood and adult life, are extremely rare and occur only in states of severe debilitation It is generally accepted that this susceptibility in the neonatal period is due to the lack of the acquired immunity which develops naturally to those pathogens which are ubiquitous within the environment This deficiency is reflected in rapidly decreasing concentrations of gamma globulin during the first few weeks of life in human infants and in the absence of an antibody response following antigenic stimulation in normal human infants At this time of life, the lymphoid tissue is quantitatively deficient and poorly organized In addition, plasma cells are absent from the bone marrow and the lymphatic tissue of the bowel and lymph nodes Studies of a baby born of an agammaglobulinemic mother revealed that this child, who ultimately was shown to be immunologically normal, could not be differentiated during the neonatal period by morphologic or immunologic criteria from patients with congenital agammaglobulinemia. We interpret this observation to be a clear reflection of the immunologically null state which characterizes the neonatal period in normal human infants. Unlike the child reported here, however, normal infants are provided a measure of protection by the passive transfer of gamma globulin and antibodies across the placenta. The normal newborn has a concentration of gamma globulin in the cord blood which regularly equals and usually exceeds that of the mother. The absence of immunologic responsiveness in the neonatal period is reflected in a steady decline of gamma globulin concentration during the first few weeks of life. The studies reported here show that the absence of immunologic responsiveness which characterizes the neonatal period in both man and rabbit has a definite morphologic correlate in the absence of plasma cells and the failure to form these cells in response to antigenic stimulation. In human beings it would appear that immunologic capability generally develops sometime during the second month of life, and that this capacity is correlated with the appearance of plasma cells in the tissues and the production of these cells in response to antigenic stimulation. In the rabbit a similar situation prevails except that the assumption of immunologic responsibility may occur before the twenty-first day of life.

The early days of the neonatal period are also those in which the various states of immunologic tolerance may be produced. The production of the phenomena of tolerance also appears to depend upon the antigens gaining access to the body during the period of immunologic unresponsiveness. Dixon's[33] recent studies show that, within certain quantitative limits, antigenically stimulated lymph node cells from adult rabbits, when transferred to the neonatal rabbit, do not result in significant production of antibody. Such cellular transfers, when carried out in mature animals, regularly result in antibody formation in both normal and irradiated recipients, and it seems that the inability to effect such transfers in newborn rabbits reflects the functional inadequacy of the neonate to accomplish certain critical steps in the synthesis of antibody globulin. From the investigations reported in this paper, it is evident that the newborn is incapable of the synthesis of circulating antibody and is unable to produce the cells that are believed to be responsible for antibody synthesis. It is not certain, however, whether the deficiency of lymphoid cells and failure to develop plasma cells is the basis for the immunologic inadequacy of the newborn human and rabbit or whether the failure to develop plasma cells is secondary to some more basic disturbance in the capacity to synthesize antibody which, in turn, is reflected in the failure of plasma cell development in response to antigenic stimulation.

In the lamina propria of the intestine and bone marrow of human infants, the complete absence of plasma cells was noted during the first weeks of life. Although our data are somewhat meager, it would appear that in man this state of immunologic unresponsiveness occasionally lasts as long as 3 to 4 months. At the opposite end of the spectrum, plasma cells have been found in the lymphoid tissues as early as the third week of life in the human material studied. This variability is reflected in the variation that we[34] and others[4-6] have observed in the period of decline of serum gamma globulin concentration during the neonatal period. The lowest level of gamma globulin reached is variable and may occur anywhere between the fourth and the sixteenth weeks after birth. In an occasional child, there appears to be a clinically significant delay in the assumption of immunologic responsibility and the gamma globulin level may fall below 100 mg./100 ml. Apparently, with these levels there is an increased susceptibility to pneumonia, septicemia, and to the development of progressive vaccinia.[17, 34, 3] We have studied several such children who later began to form gamma globulin satisfactorily and were shown to be immunologically normal. The stimuli provided by an extrauterine existence might be held responsible for the development of immunologic maturation. However, the persistence of an agammaglobulinemic state for 42 days, even in the face of intensive antigenic stimulation, in the immunologically normal child born of an agammaglobulinemic mother suggests that the antigenic stimuli of the environment do not serve to initiate the normal capacity for an immune response during the later part of the neonatal period.

In rabbits, the relatively mild antigenic stimulus afforded by a saline solution of BSA did not result in an immune response in animals that were younger than 28 days at the time of injection. When a more intense antigenic stimulus was given by the use of a water in oil emulsion containing *Mycobacterium butyricum,* a definite morphologic response was evident as early as the sixteenth day, and circulating antibody could be demonstrated by the nineteenth day of life. These studies, then, are interpreted as defining for the rabbit the limits during which even the most intensive antigenic stimulation does not result in an immune response defined either morphologically or by the development of circulating antibody. Further studies of antibody production such as those of Sterzl and Hrubesova[3] may contribute to our understanding of the nature of the immune response in man and animals. It appears likely that an essential phase of antibody globulin synthesis cannot proceed during this period but becomes possible a few weeks later. The nature of this essential step and the mechanisms controlling it may be of critical significance for a complete understanding of the immune response.

The parallel between agammaglobulinemic patients and the neonate is indeed striking. Repeated efforts to demonstrate a factor inhibiting antibody and gamma globulin formation in the blood and tissues of patients with both congenital and acquired agammaglobulinemia have been fruitless.[17] Dixon's studies[33] would indicate that inhibitors do not account for immunologic unresponsiveness in the neonatal period. These states, then, are indistinguishable by the available morphologic and immunologic criteria, and it is only the normal maturation of immunologic potential during the later part of the neonatal period that allows differentiation of the immunologically normal child from the agammaglobulinemic patient. It seems probable, as Martin has suggested,[38] that congenital agammaglobulinemia represents a disease state resulting from the indefinite persistence of the immunologically null period which is present in all infants during the first several weeks of extrauterine existence.

SUMMARY

Detailed investigations of an immunologically normal child born to a woman with acquired agammaglobulinemia have provided an opportunity to observe the maturation of immunologic characteristics during the newborn period. A serial study of the gamma globulin concentration by an immunochemical technique revealed the gamma globulin concentration to be below 11 mg. per 100 ml. until the sixth week of life when gamma globulin began to accumulate rapidly in the serum. Although intensive antigenic stimulation was provided once each week during the first 2 months of life, antibody did not appear in the serum until the sixty-seventh day of life. Subsequently this child's gamma globulin concentration rose to normal levels, and the child has maintained those levels found in normal children. Serial bone marrow and lymph node biopsies did not reveal plasma cell production during the neonatal period. The capacity to produce plasma cells, however, developed concurrently with the capacity to synthesize gamma globulin and antibody.

Examination of bone marrow biopsies from 20 normal infants during the first week of life has shown that plasma cells are normally absent from the marrow at this time. The development of the capacity to form plasma cells during the neonatal period has been revealed by a study of the lamina propria of the ileum and appendix of children of various ages. Plasma cells are not present in these sites at birth or in the first few weeks of life, but appear in this location between the fourth and the sixth weeks of life. The majority of children 6 months of age and older have large numbers of plasma cells in these areas.

The development of plasmacytosis in response to even the most intense antigenic stimulation does not occur prior to the age of 16 days in the rabbit. Precipitating antibody did not appear in the serum in rabbits less than 19 days of age regardless of the intensity of antigenic stimulation. The morphologic changes occurring in the lymph nodes draining the site of injection of BSA in Freund's complete adjuvant at an age prior to the development of immunologic capacity and plasma cell formation are described.

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ABNORMAL URINARY AMINO ACID PATTERNS IN ACUTE INTERMITTENT PORPHYRIA

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ACUTE intermittent porphyria remains a metabolic and physiologic enigma. Although much has been learned about the porphyrin metabolites that appear in this disease, the fundamental biochemical defect and the pathogenesis of the signs and symptoms are not known. Abnormal amino acid excretion has been observed in rabbits given drugs that produce an experimental form of porphyria.[1] Hence, as a vague exploratory gesture, the blood of a patient with acute intermittent porphyria (during remission) was analyzed for its amino acid content by two-dimensional chromatography.[2] There appeared to be no abnormalities apart from an increase in the threonine concentration, and the latter seemed of dubious significance.

More recently, one of us (R. M. H.), intrigued by the occasional finding of a faintly "positive" Watson-Schwartz test for porphobilinogen in patients not having porphyria, suggested that the chromatographic analysis of such a urine be compared with the urine of a patient with acute intermittent porphyria. The chromatogram of the "false positive" patient's urine appeared to be quite normal as compared with 50 normal chromatograms previously done in the same laboratory. In the case of the patient with porphyria, however, there were striking abnormalities, including moderate elevation in threonine and cysteic acid and more phenylalanine than had previously been found in any specimen other than the urine of patients with phenylpyruvic oligophrenia. It was decided, therefore, to screen the blood and urine of all available patients with porphyria for amino acid abnormalities.

MATERIAL AND METHODS

Twelve patients with acute intermittent porphyria were located (Table II). Four of them, 3 women and 1 man, were in acute relapse, with abdominal pain or neurologic manifestations. The other 8, all women, were at the time of testing either asymptomatic or having only mild neurosis-like complaints. All but one of the patients had experienced typical symptomatic bouts of acute intermittent porphyria, and all of them had moderate to large amounts of porphobilinogen in the urine at the time of these experiments. In addition, one patient with mixed porphyria was tested.

One or two fasting venous blood specimens were obtained from each patient, using dried heparin as an anticoagulant. The first morning urine specimen following the usual nocturnal fast was obtained a total of 28 times from the 13 patients. Color photographs

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of 125 blood specimens and 43 urine specimens from normal healthy individuals were available. In order to assure contemporary comparison, however, an additional 10 normal volunteers were used as controls in these experiments.

The blood specimens were deproteinized with absolute alcohol, an aliquot of filtrate spotted on filter paper, and two dimensional chromatograms made using tert butyl alcohol and phenol as the solvents and ninhydrin as the color developer.[2] All urine specimens were deproteinized with 75 per cent ethyl alcohol, desalted by ion exchange (Dowex 50 X 8 hydrogen form) and evaporated or diluted to a constant of 40 μg of creatinine per 25 μl of solution. The chromatograms were then processed in the same manner used for blood. This method is roughly semiquantitative, and in this laboratory the following amino acids have been regularly identified in normal blood: leucine and isoleucine (1 spot), valine, phenylalanine, tyrosine, α alanine, threonine, serine, glycine, glutamic acid, lysine, cysteic acid, and glutamine. Less often the following are found: proline, α amino n butyric acid, and arginine. Special stains have been required to demonstrate others. In the normal urine all of the above listed amino acids have usually been seen except lysine, proline, and arginine (Fig. 1). In addition, the following have usually been found: β alanine, asparagine, and lysine superimposed upon histidine. Beta aminoisobutyric acid is identified in some urine specimens as a spot under that of valine which becomes deep purple after spraying with ninhydrin and heating to 110° F.[3] It is not affected by acid hydrolysis. This spot changes to deep blue upon dipping in 1 per cent sodium bicarbonate and is not completely washed from the paper by rinsing in water for 3 minutes. Homocystine and aspartic acid are also sometimes found in normal urine. Unidentified spots found in normal urine chromatograms with Rf values as indicated are listed in Table I.

TABLE I. RF VALUES FOR UNIDENTIFIED SPOTS FOUND IN NORMAL URINE CHROMATOGRAMS*

SPOT	Rf VALUES	
	BUTANOL	PHENOL
A	0.44	0.91
B	0.28	0.78
C	0.38	0.44
D	0.19	0.22
E	0.14	0.24
F	0.16	0.35

*Spot B may be sarcosine. Sarcosine standard gave Rf values of 0.78 and 0.30 in phenol and butanol, respectively.

RESULTS

Since the methods employed are only semiquantitative, questionable departures from normal were ignored, and only clear and obvious abnormalities recorded.

All of the blood chromatograms appeared to be within normal limits except for 2 of 3 specimens obtained from one patient (F. H.). In these 2 instances there were slight to marked increases in the concentrations of phenylalanine, glutamic acid, threonine, and lysine. Dr. S. Wright of the U.C.L.A. Department of Pediatrics kindly took two blood specimens from this patient to Dr. R. Lippman at the California Institute of Technology, where quantitative phenylalanine determinations were done by the method of Udenfriend and Cooper.[4] Dr. Lippman found concentrations of 10 and 3 mg. per cent in 2 specimens which by the chromatographic technique appeared to show a marked increase and a normal concentration of phenylalanine, respectively.

In most of the urine chromatograms there were two yellow spots before the application of ninhydrin. One of these spots had the following Rf values

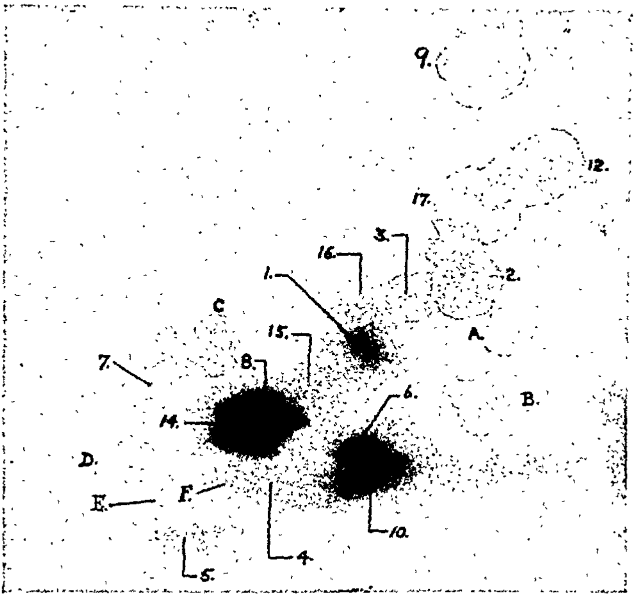

Fig. 1.—Black and white photograph of normal urine chromatogram. Colors present in original paper help in estimating concentrations and also in distinguishing some overlapping spots. Glycine, for example, is brown, and serine is purple. Numbers indicate identity of spots as follows: 1, α-alanine; 2, α-amino-n-butyric acid; 3, β-alanine (slate blue) or δ-amino levulinic acid (yellow); 4, asparagine; 5, cysteic acid; 6, glutamine; 7, glutamic acid; 8, glycine; 9, leucine and isoleucine (inseparable); 10, lysine and histidine; 12 phenylalanine; 14, serine; 15, threonine; 16, tyrosine; 17, valine and β-aminoisobutyric acid. A, B, C, and D are unknown. A (purple) overlaps proline (yellow). B may be sarcosine.

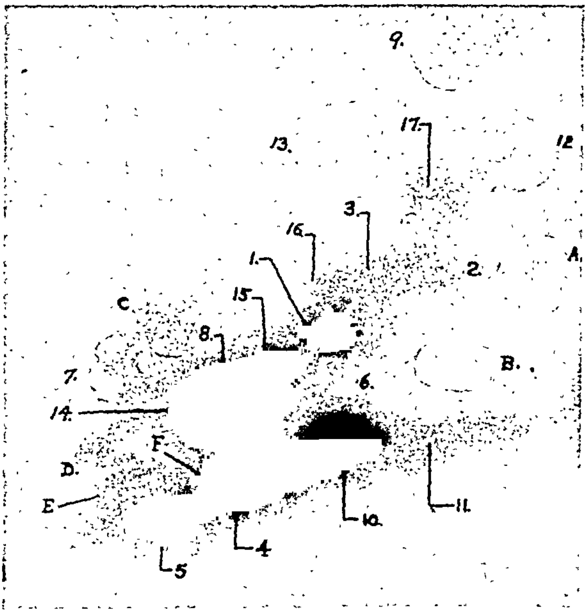

Fig. 2.—Chromatogram of urine from Patient F. H. with exacerbation of acute intermittent porphyria. Besides spots listed under Fig. 1, the following are present: 1, methyl histidine and carnosine and 13, porphobilinogen. In addition, the following are present in abnormally high concentration: phenylalanine (12), β-aminoisobutyric acid (17), δ-amino levulinic acid (3), threonine (15), glycine (8), cysteic acid (5), and lysine and/or histidine (4).

phenol 0 56, and butanol 0 64 The other yellow spot had phenol and butanol Rf values of 0 64 and 0 43, respectively The first of these was, in all probability, porphobilinogen and the second δ amino levulinic acid Porphobilinogen and δ amino levulinic acid were isolated from the urine by ion exchange[5] On paper chromatograms these substances appeared with Rf values identical to the yellow spots described above

The results of the chromatographic studies on the urines from patients with porphyria are summarized in Table II and illustrated in Fig 2 All of the specimens were abnormal, but some of them contained grossly excessive amounts of many amino acids while others contained only slightly increased amounts of 1 or 2 amino acids In the 28 urine specimens the number of times the substances listed were found in high concentration is indicated as follows α alanine 3, δ amino levulinic acid 26 cysteic acid 24, glutamine 2, glycine 9, leucine isoleucine 2, phenylalanine 15, porphobilinogen 24, serine 6, threonine 13, beta aminoisobutyric acid 14 Some of the unknown spots were also present in high concentration as listed in Table II

No abnormalities were seen in the chromatographic analysis of the blood and urine specimens of the patient with mixed porphyria except for a moderately increased urinary concentration of δ amino levulinic acid

DISCUSSION

The striking differences between the normal urines and those from patients with acute intermittent porphyria clearly indicate abnormalities in amino acid excretion That the blood amino acids are sometimes abnormal in this disease is not certain from these experiments, but is suggested by the findings in one patient

The urinary abnormalities are difficult to quantitate or even to define Since some of the patients were too sick to follow a strict dietary regimen food and fluid intake were not controlled Under these circumstances comparisons based upon timed aliquots of urine would be of dubious value, since the amount of urine produced in a given period of time would be influenced by the degree of hydration, salt consumption, and many other variable factors Hence 40 μg of urinary creatinine was arbitrarily used as the standard to which comparisons of amino acid excretion could be referred Creatinine excretion is related chiefly to the size of the muscle mass It is possible that this means of standardizing the quantity of urine used for comparing amino acid pattern is not a desirable one Not enough is yet known about normal amino acid excretion to be sure a priori that any particular standard would be better than another Nevertheless it can at least be concluded that the ratio of amino acid to creatinine excretion is quite different in normal individuals and patients with porphyria This fact, of course is only the beginning of an inquiry into amino acid metabolism in porphyria

Another standard of comparison is inherent in these experiments It is the relationship between the density of amino acid spots on a given paper Thus the urinary aliquot of one patient contained normal amounts of all of

TABLE II. SUBSTANCES FOUND IN HIGH CONCENTRATION IN URINE FROM PATIENTS WITH PORPHYRIA. NUMBERS AND LETTERS REFER TO SUBSTANCES AS INDICATED IN LEGENDS OF FIGS. 1 AND 2 AND IN TABLE I. THE NUMBER "3" IN THIS TABLE ALWAYS REFERS TO δ-AMINO-LEVULINIC ACID AND "17" TO β-AMINO-ISOBUTYRIC ACID

DATE	PATIENT	CHROMATOGRAPHIC SPOTS FOUND IN MODERATELY HIGH CONCENTRATION	CHROMATOGRAPHIC SPOTS FOUND IN VERY HIGH CONCENTRATION	CLINICAL CONDITION
12/4	H. T.	1,3,5,12,13,17, and F	B	Agitated, whining, odd complaints
1/6	H. T.	3,12,13,17	None	Same as on 12/4
12/31	F. H.	6,8	3,5,12,13,15,17, and F	Acutely and severely ill, neurologic signs, agitation
1/20	F. H.	3 and 13	5,8,10,12, and 15	As on 12/31
2/3	F. H.	3,5,8,10,12,13, and 14	15	Cataleptic
2/6	F. H.	5,6,7,8	3,13,15	Slightly improved over 2/3
2/8	F. H.	3,8,13,15	5	Striking improvement
2/10	F. H.	1,3,8,13,15	5,12,17,A	Almost complete remission
2/11	F. H.	3,5,8,13,15	9,14,17,A	Same as on 2/10
3/1	F. H.	3,5,8,15	12,13,14,17,A	Same as on 2/10
3/18	F. H.	3,5,12,13,17	None	Whining, weak
1/8	L. R.	5,13	3	Asymptomatic
1/16	L. R.[1]	5,15	3,13	Severe abdominal pain and emotional disturbances
1/20	L. R.[1]	3,5,13,15	None	Somewhat improved over 1/16
1/3	J. M.	3,5,12,13	None	Complete remission
2/7	J. M.	3,5	13	Complete remission
1/30	M. B.	3,12,13	10	Few odd "psychoneurotic" complaints
1/31	J. D.	5,12,15	3,13,17	Muscle weakness and other neurologic signs
2/3	J. D.	5,14	3,13,17	Same as on 1/31
2/5	J. D.	5,12,15	3,13	Same as on 1/31
2/9	J. D.	5,14	3,13,17	Same as on 1/31
3/4	N. S.	3	None	Mixed porphyria, abdominal pain and tenesmus but emotionally stable
3/17	J. M.[1]	3,13	None	Asymptomatic
3/18	U. E.	5,12,15	3,13	Asymptomatic, taking BAL
3/20	T. D.	5,9,17	1,7,8,10,12,14	Asymptomatic
5/20	E. S.	12,17	5	Neurasthenic
6/2	F. A.	3,5	17	Acute abdominal pain
6/5	F. A.	3,5,13	[illegible]	Same as on 6/2

the amino acids usually found except for obviously excessive amounts of cysteic acid Even if the creatinine standard was misleading, it would appear that either the excretion of cysteic acid was increased or the excretion of the other amino acids was diminished

Poor diet in some of the very sick patients may have been responsible for some of the abnormalities seen However, this possibility seems unlikely because similar abnormalities were found in asymptomatic individuals with porphyria who were eating normally

Delta amino levulinic acid, a precursor of the porphyrin ring has previously been found in the urine of patients with porphyria[5] We are not aware of other previously reported abnormalities in amino acid metabolism in patients with porphyria

It is conceivable that the abnormalities reported here are secondary to alterations in the metabolism of porphyrins but it seems more likely that some more basic biochemical defect is responsible for the abnormal porphyrin metabolites as well as the abnormal amino acid excretion Goldberg and Rimington[1] have reported abnormal amino acid excretion in rabbits suffering from drug induced porphyria but the urinary amino acid pattern of the rabbits excreting the most porphobilinogen was more nearly like that of normal rabbits This observation might suggest alternate metabolic consequences of an underlying enzyme deficiency induced by the drug The findings here were not the same as those reported by Goldberg Nevertheless the drug induced disease in rabbits, even if analogous to human porphyria might be expected to create different metabolic effects

More precise observations will be necessary before these amino acid peculiarities can be traced to their source It would seem worth while to do so since the symptoms and signs of acute intermittent porphyria are not well correlated with the excretion of porphobilinogen As is shown in Table II there appeared to be little if any, tendency for the clinical condition of the patients in this series to be associated with the degree of amino acid abnormalities but the number of observations are too few to justify conclusions

SUMMARY

Abnormal urinary amino acid excretion was found in 12 patients with acute intermittent porphyria One patient also had abnormal quantities of a few amino acids in the blood The cause of these findings is not known

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A COMPARISON OF ERYTHROMYCIN WITH OLEANDOMYCIN IN COMBINATION WITH OTHER ANTIBIOTICS AGAINST HOSPITAL STAPHYLOCOCCI

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THIS study was prompted by the recent controversy concerning the efficacy of antibiotic combinations in the treatment of staphylococcal infections. Some authors[1-5] have reported certain antibiotic combinations to show potentiation or even synergism of antibacterial effects and also to retard the emergence of resistant strains of staphylococcus. Others have not confirmed these findings and have outlined objections to antibiotics combined in mixtures with fixed ratios.[6-8] We have attempted, by the tube dilution and agar diffusion methods, to evaluate some paired combinations of 12 common antibiotics against 30 hospital strains of coagulase-positive hemolytic *Staphylococcus aureus*. Additive antibacterial effect was frequent but synergism was rare.[9]

In 1956, English and co-workers[1] reported synergistic activity with Sigmamycin,[*] a physical mixture of one part tetracycline and two parts of oleandomycin, a new antibiotic that is very similar to erythromycin. Growth of 8 out of 21 selected strains of antibiotic-resistant[†] staphylococcus was inhibited by Sigmamycin in a concentration significantly less than required with either oleandomycin or tetracycline alone. English also found a high degree of protection for 4 days against a usually fatal staphylococcal infection in mice. The protection afforded was proportional to the size of a single oral dose of Sigmamycin and greater than could be expected through the addition of the protective effects of oleandomycin and tetracycline.

Similar results were reported by English and his co-workers[1] with 3 antibiotic-resistant strains of staphylococcus when much larger doses of Sigmamycin were given. Unfortunately the data from each of their individual experiments were not given. Averages of 8 experiments were reported in each instance and intragroup variations were not stated. A statistical evaluation of the data indicated significant enhancement of protection by Sigmamycin.

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[*] Oleandomycin, Sigmamycin (a physical mixture of 67 per cent tetracycline and 33 per cent oleandomycin), oleandopen or AP (an oleandomycin salt, 67 per cent oleandomycin and 33 per cent penicillin G by weight) and fortified oleandopen or APG (110 mg. AP with 73 mg. added penicillin G) were generously supplied through the courtesy of Chas. Pfizer & Co., Inc., Brooklyn, N. Y.

[†] Garrod[8] was unable to confirm the antibiotic resistance and Sigmamycin synergism with one of these strains in his laboratory.

The level of in vitro resistance of the 3 antibiotic resistant strains of staphylococcus was not given Tetracycline alone had no protective activity against these 3 strains in mice, although tetracycline did enhance the protection afforded by large doses of oleandomycin The enhancement of protection was significant only with very large doses of Sigmamycin

Carter and Maley[2] used Sigmamycin in 125 patients, most of whom had upper respiratory infections The drug was well tolerated and nontoxic and seemed to be effective in a variety of infections

Winton and Chesrow[3] reported the use of Sigmamycin in 50 patients with a variety of infections The infections were controlled in all but 3 cases The only side effect noted was soft stools in 3 patients

In another clinical report, La Caille and Prigot[1*] used Sigmamycin in 79 cases, including 48 patients with cellulitis and 31 patients with other infections of soft tissues The clinical response was excellent and side effects were rare No data were given with regard to bacterial resistance

The oleandomycin salt of penicillin G, oleandopen (compound AP), was studied by Hobby and co workers[4] Eighteen strains of staphylococcus were selected which were highly resistant to erythromycin and considerably more susceptible to oleandomycin These strains were also resistant to penicillin, yet all but one were more susceptible to compound AP than to either penicillin G or oleandomycin The rate of emergence of resistance to compound AP was retarded when compared with penicillin G and oleandomycin separately with two susceptible and one penicillin resistant strains of staphylococcus Similar results were obtained with mechanical mixtures of oleandomycin and penicillin G Compounds AP and APG (AP fortified with penicillin G) protected mice infected with both penicillin susceptible and resistant strains of staphylococcus The strains used were all oleandomycin susceptible

Blood levels of antibacterial activity were measured in human subjects given compounds AP and APG by Payne and co workers[5] The antibacterial activity was maintained for a few hours longer in the blood with compounds AP and APG than with potassium penicillin G Seven patients with various infections improved when treated with compound AP

Jones and Finland[6] studied antibacterial activity of the blood in 6 normal men after oral administration of oleandomycin penicillin G, and compounds AP and APG against susceptible staphylococcus Penicillin G was the most active and oleandomycin was the least effective of these antibiotics The compounds AP and APG showed no enhanced activity In a subsequent study[7] the same authors compared the antibacterial activity of 3 antibiotic mixtures 2 parts of tetracycline with 1 part of either oleandomycin erythromycin, or spiramycin They found the most active combination to be tetracycline erythromycin However, of 34 strains of staphylococcus tested only 2 were more sensitive to the mixture of tetracycline erythromycin than to the most active of the components in the combination, most of their strains were tetracycline resistant Jones and Finland also found that these antibiotic combinations produced no greater antibacterial activity in the plasma of normal adults after a single oral dose than after an equivalent dose of the most active of the antibiotics in the combinations. Erythromycin was much more potent in vitro than oleandomycin or spiramycin against most strains of staphylococcus and produced much greater antistaphylococcal plasma activity after oral administration to human beings.

Garrod[6] found no enhanced or synergistic effect with Sigmamycin against 9 strains of staphylococcus when compared with oleandomycin and tetracycline in broth. Repeated exposure of 6 strains of susceptible staphylococcus to Sigmamycin led to an emergence of significant resistance to tetracycline and to a lesser extent to Sigmamycin but not to oleandomycin. The addition of tetracycline seemed to retard resistance to oleandomycin but the reverse was not true. Garrod tested 45 strains of erythromycin-resistant staphylococcus obtained from various hospitals and found 29 susceptible to oleandomycin. Strains fully resistant to the closely related antibiotics (erythromycin, oleandomycin, and spiramycin) showed a uniform population of resistant colonies. Strains which were resistant to only 1 or 2 of the 3 antibiotics produced less than one colony in 100 which was fully resistant. Furthermore, resistance of the "dissociated" type to erythromycin only was unstable, requiring the continued presence of erythromycin. Dissociated resistant strains became resistant to oleandomycin and spiramycin, as well as to erythromycin, in the presence of moderate concentrations of erythromycin, a curious type of antibiotic interaction resembling but not identical with the phenomenon of drug dependence. Garrod also induced resistance in staphylococci and found that erythromycin induced partial cross-resistance to oleandomycin but little to spiramycin. Strains rendered resistant to spiramycin also became resistant to oleandomycin and erythromycin as well.

Andrieu and associates[13] tested 324 strains of staphylococcus isolated from clinic patients in 1953 and 1954 and found them all susceptible to the erythromycin group of antibiotics although erythromycin was more active than oleandomycin and oleandomycin was more effective than carbomycin or spiramycin, the least active member of the group. Of 126 strains of staphylococcus from patients in the hospital in 1956, about 8 per cent were resistant to erythromycin and 10 per cent resistant to oleandomycin. They induced resistance to each of the 4 antibiotics in 3 strains of staphylococcus and demonstrated almost complete cross-resistance to the 4 antibiotics in one strain made resistant to oleandomycin. The same strain showed less cross-resistance when erythromycin resistance was induced. Studies with 36 strains of staphylococcus were made by paper strip-agar diffusion comparing the antibacterial activity in pairs of erythromycin, oleandomycin, carbomycin, spiramycin, penicillin, streptomycin, tetracycline, chloramphenicol, and novobiocin. "Synergism" was reported to occur most frequently with oleandomycin combined with streptomycin, penicillin, or novobiocin. Synergism was found with similar frequency with erythromycin in association with penicillin or streptomycin. Unfortunately, the authors did not define the word synergism as used by them.

In the present study the susceptibility of 30 strains of hospital staphylococcus to erythromycin, oleandomycin, Sigmamycin, oleandopen (AP), and fortified oleandopen (APG) was measured by the tube dilution method.[9] The bactericidal end point for each antibiotic preparation was elicited by subculture of each broth tube to an agar plate. The combined effect of pairs of each of these antibiotic compounds upon the 30 strains of hospital staphylococcus was measured by a paper strip agar diffusion method.[9] Penicillin, tetracycline, chloramphenicol, neomycin, bacitracin, vancomycin, and novobiocin were also tested for combined effects with members of the erythromycin group. The influence of paired antibiotic combinations upon bactericidal activity was measured by preparing replica plates from the original agar plates with velveteen stamps by the method of Lederberg.[9] As in our previous study, synergism of antibacterial effects with a pair of antibiotics was recorded only when the junction of the two antibiotic inhibition zones was expanded along a straight line. No cases of synergism were observed in the present study.

MATERIALS AND METHODS

The materials and methods used were identical to those described in a previous study.[9] The concentration of the oleandomycin salts and mixtures in the paper strips for agar diffusion was 100 μg per milliliter. The antibiotics were freshly dissolved in sterile distilled water.

TABLE I. THE BACTERIOSTATIC CONCENTRATION OF 7 ANTIBIOTICS AGAINST 30 HOSPITAL STRAINS OF STAPHYLOCOCCUS AUREUS (TUBE METHOD)

ANTIBIOTIC	MICROGRAMS OR UNITS PER MILLILITER									
	0.39	0.78	1.56	3.12	6.25	12.5	25	50	100	100
Erythromycin	3*	*16*	5	1	4	0	0	0	0	1
Sigmamycin	11	*4*	3	5	2	0	0	1	4	0
AP	11	2	*11*	1	0	0	0	5	0	0
APG	9	3	*4*	9	0	0	0	2	3	0
Oleandomycin	2	2	6	*15*	0	0	0	0	5	0
Tetracycline	9	0	1	0	1	2	*6*	5	3	3
Penicillin G	10	0	0	0	0	0	0	0	3	*17*

Median values are indicated by italics.
*Each number refers to the number of strains inhibited.

RESULTS

The bacteriostatic activity of the erythromycin group of antibiotics is given in Table I. Erythromycin was the most effective antibiotic of the group tested. Sigmamycin showed considerably more activity than did either of its two components, oleandomycin and tetracycline. Those antibiotic mixtures which contained penicillin (AP and APG) were slightly more active than oleandomycin and much more active than penicillin alone. It should be noted that the strains of staphylococci tested were isolated late in 1954 and most of them were highly resistant to penicillin and tetracycline.

The bactericidal activity (tube method) of these same antibiotics is given in Table II. Erythromycin again appeared to be the most active antibiotic of this group. The oleandomycin combinations appeared only slightly

TABLE IV. ANTAGONISTIC ANTIBACTERIAL ACTIVITY OF 12 ANTIBIOTICS WITH 30 HOSPITAL STRAINS OF STAPHYLOCOCCUS AUREUS (BY AGAR DIFFUSION WITH REPLICA PLATING)

	BACTERIOSTATIC COMBINATIONS (ORIGINAL PLATE)				BACTERICIDAL COMBINATIONS (REPLICA PLATE)				
ANTIBIOTIC	O	Si	APG	AP	AP	APG	Si	O	
Si	0							0	Si
APG	0	0					5	5	APG
AP	0	0	0			0	7	7	AP
No	0	0	0	0	0	3	5	4	No
E	0	0	1	1	7	5	0	0	E
V	0	0	0	0	0	0	0	8	V
T	0	1	0	0	3	4	0	0	T
P	0	0	0	0	0	0	3	3	P
C	0	0	0	0	0	5	0	0	C
N	0	0	0	0	0	0	0	0	N
B	0	0	0	0	0	0	0	0	B

Note: For key to abbreviations see Table III.

The number given indicates the number of strains out of 30 showing antagonism of antibacterial activity with a pair of antibiotics

TABLE V. COMBINED ANTIBACTERIAL EFFECTS OF 38 OLEANDOMYCIN COMBINATIONS AGAINST 30 HOSPITAL STRAINS OF STAPHYLOCOCCUS AUREUS (BY AGAR DIFFUSION WITH REPLICA PLATING)

	NO EFFECT		ADDITION		ANTAGONISM	
STRAIN	ORIGINAL PLATE	REPLICA PLATE	ORIGINAL PLATE	REPLICA PLATE	ORIGINAL PLATE	REPLICA PLATE
1	17*	14	21	17	0	7
2	17	22	21	21	0	12
3	22	17	16	26	0	0
4	23	17	15	19	0	2
5	21	17	17	20	0	1
6	21	12	17	25	0	1
7	19	22	19	15	0	1
8	22	18	16	18	0	2
9	14	13	24	21	0	4
10	16	25	19	13	3	0
11†	29	29	9	9	0	0
12	11	11	27	27	0	0
13†	25	28	13	10	0	0
14	11	14	27	19	0	5
15†	29	28	9	10	0	0
16	24	16	14	20	0	2
17†	25	25	13	13	0	0
18	25	13	13	17	0	8
19	9	11	29	27	0	0
20	19	13	19	25	0	0
21	20	16	18	20	0	2
22†	30	31	8	7	0	0
23	15	4	23	23	0	11
24	11	6	27	31	0	1
25	14	9	24	29	0	0
26	19	17	19	19	0	2
27	16	10	22	17	0	11
28	14	9	24	24	0	5
29	18	12	20	25	0	1
30	11	11	27	27	0	0

*Each number refers to the number of cases of combined effect out of a possible total of 38.

†These strains were highly resistant by the tube method to oleandomycin, AP, APG, Sigmamycin, and many of the other antibiotics tested. It will be noted that none of these strains showed antagonism and all showed less addition than the more susceptible strains. Other strains showing less resistance had a higher incidence of addition and antagonism. This demonstrates that for an organism to react favorably or unfavorably to a combination it must be susceptible to the antibiotics used. No instances of synergism were found.

strains of staphylococcus (Nos 11, 13, 15, 17, and 22) highly resistant to the oleandomycin group, as well as to other antibiotics The resistant strains, as might be expected, showed less addition of antibiotic activity than the more susceptible strains Furthermore, the highly resistant strains showed no antagonism between antibiotics Three susceptible strains (Nos 2, 23, and 27) showed a higher incidence of antagonism. Table VI again demonstrates that resistant strains of staphylococcus showed less addition of antibiotic activity than did the susceptible strains Likewise, resistant strains showed less antagonism of antibiotic bactericidal effects

TABLE VI ANTIBACTERIAL ACTIVITY OF 4 OLEANDOMYCIN COMBINATIONS AND 8 OTHER ANTIBIOTICS IN PAIRS AGAINST RESISTANT AND SUSCEPTIBLE STRAINS OF STAPHYLOCOCCUS AUREUS

ANTIBIOTIC	NUMBER OF STRAINS	ORIGINAL PLATE			REPLICA PLATE		
		NO EFFECT (PER CENT)	ADDITION (PER CENT)	ANTAGONISM (PER CENT)	NO EFFECT (PER CENT)	ADDITION (PER CENT)	ANTAGONISM (PER CENT)
*A Resistant**							
Oleandomycin	5	60†	40	0	58	42	0
Sigamycin	7	81	19	0	77	18	5
AP	5	71	29	0	71	29	0
APG	5	69	31	0	78	22	0
B Susceptible							
Oleandomycin	25	40	60	0	28	62.2	9.8
Sigamycin	23	41.5	58.1	0.4	32.4	61.3	6.3
AP	25	43.6	56	0.4	29.8	60	10.2
APG	25	46.2	53.4	0.4	38.9	51.3	9.8

*Resistant to 6.25 µg or more per milliliter of antibiotic in column 1 by the tube dilution method

†Each number represents the per cent of cases showing addition antagonism or no effect upon antibacterial activity when tested in combination with another antibiotic

TABLE VII EFFECT OF ANTIBIOTIC RESISTANCE UPON ACTIVITY OF OLEANDOMYCIN COMBINATIONS AGAINST HOSPITAL STAPHYLOCOCCI

ANTIBIOTIC	NUMBER OF STRAINS	ORIGINAL PLATE		REPLICA PLATE	
		NO EFFECT (PER CENT)	ADDITIVE (PER CENT)	NO EFFECT (PER CENT)	ADDITIVE (PER CENT)
1 Sigmamycin					
Tetracycline					
Susceptible	10	0	100	10	90
Resistant	20	90	10	90	10
2 Oleandopen (AP)					
Penicillin G					
Susceptible	10	50	50	30	70
Resistant	20	75	25	70	30
3 Fortified oleandopen (APG)					
Penicillin G					
Susceptible	10	20	80	30	70
Resistant	20	70	30	65	35

Note None of these combinations proved antagonistic or synergistic

In Table VII it is shown that oleandomycin had enhanced bacteriostatic and bactericidal effects against susceptible staphylococci upon the addition of tetracycline or penicillin G Additive potentiation was rarely seen with resistant strains. This confirms earlier reports[6-10] that antibiotics in pairs have little or no combined effect on bacterial growth if the strain of staphylococcus is resistant to either antibiotic.

DISCUSSION

The present studies confirm the fact[6-8] that erythromycin is much more effective than the closely related antibiotic, oleandomycin, in retarding the multiplication of staphylococci. *Jones and Finland*[6] *have also shown that* erythromycin produces greater antistaphylococcal activity in the blood of human beings after a single oral dose. Erythromycin was far more active than any of the oleandomycin combinations, particularly if subcultures of broth tubes were examined for bactericidal effect. Even in high concentrations oleandomycin failed to kill most strains of hospital staphylococcus.

Sigmamycin, a mixture of one part oleandomycin and two parts tetracycline, was more active than either component alone. The potentiation measured by agar diffusion was additive rather than truly synergistic, as claimed by English and associates.[1] Furthermore, potentiation was seldom seen with strains of staphylococcus resistant to tetracycline, which are now frequent in hospitals.

The oleandomycin salt of penicillin G (oleandopen or AP) was more actively bacteriostatic than either oleandomycin or penicillin alone. Again the potentiation was additive in behavior rather than truly synergistic. Similar results were obtained with oleandopen fortified with additional penicillin G (APG). In neither case was there enhanced activity against penicillin-resistant strains.

Observations on cross-resistance to erythromycin and oleandomycin in hospital staphylococci in general tend to confirm the greater activity of erythromycin. One strain of staphylococcus which was naturally erythromycin-resistant, however, was susceptible to oleandomycin. Five strains resistant to oleandomycin were inhibited by erythromycin. Two strains in which erythromycin resistance was induced became much more resistant to oleandomycin. Yet after induction of resistance to oleandomycin, erythromycin resistance appeared in only one of the two strains. Others[10, 11] found that strains in which oleandomycin resistance was induced were regularly cross-resistant to erythromycin. Garrod[8] showed, on the contrary, that staphylococci naturally resistant to erythromycin, although often highly resistant to oleandomycin, spiramycin, and other antibiotics of the erythromycin group, may in some cases be resistant only to erythromycin. With the latter type of "dissociated" strains, Garrod[8] demonstrated that erythromycin in moderate concentrations permitted the staphylococci to grow in the presence of oleandomycin. The dissociated strains were not dependent upon erythromycin but growth stimulation was dependent upon the concentration of erythromycin in oleandomycin media.

The present work demonstrated neither antagonism nor synergism of action between erythromycin and oleandomycin against either susceptible or resistant strains of staphylococcus. These two antibiotics frequently had additive antibacterial activity but with resistant strains only the most active antibiotic was effective, and this usually was erythromycin.

Potentiation of the bacteriostatic effects of oleandomycin was seen much more frequently upon the addition of bacitracin, chloramphenicol, and erythromycin than with tetracycline in these resistant hospital strains of staphylococcus. This is to be expected. It is pertinent that of 179 strains of coagulase-positive staphylococcus isolated from the blood or infected lesions of patients *in this hospital in 1957, the percentage resistant to various antibiotics* (disk method) was as follows: tetracycline 69, penicillin 68, streptomycin 66, erythromycin 28, chloramphenicol 18, novobiocin 1, and neomycin 0.6.

Previous studies[9] with the same 30 hospital strains used in the present work showed antagonism of the bactericidal action of novobiocin by several commonly used antibiotics. The present investigations revealed antagonism of this type most frequently with Sigmamycin, especially in combination with oleandopen (AP), fortified oleandopen (APG), and novobiocin. Antagonism of bactericidal activity was fairly frequent with oleandomycin combinations, particularly with vancomycin and oleandopen (AP). Antagonism with erythromycin combinations was much less frequent.[9]

Antagonism of antibacterial effects occurred only with susceptible strains of staphylococcus (Table VI) and seemed to be characteristic of certain of these cultures (Table V). Additive potentiation of antibiotic effects was seen frequently with susceptible strains but rarely with resistant strains (Tables V, VI and VII). This observation does not support the conclusion of English and his group[1] that synergistic potentiation occurs with Sigmamycin against resistant staphylococci. It may be important that they used selected strains of staphylococcus and their broth method of in vitro measurement permits a considerable variation in results when repeated or compared with results obtained by agar diffusion.

SUMMARY AND CONCLUSIONS

1. Thirty hospital strains of coagulase-positive hemolytic *Staphylococcus aureus* were exposed to individual antibiotics of the erythromycin group: erythromycin, oleandomycin, oleandopen (AP), fortified oleandopen (APG), and Sigmamycin. These strains were then tested against pairs of all of these antibiotics and bacitracin, chloramphenicol, tetracycline, penicillin, neomycin, *vancomycin, and novobiocin*. The tube dilution technique was used to measure bacteriostasis by single antibiotics and subculture of the clear tubes to measure bactericidal end points. The paper strip agar diffusion method was used to test the bacteriostatic effect of antibiotic pairs. Replica plates were prepared to record the bactericidal action of antibiotic pairs.

2. The most active drugs as measured by bacteriostatic effect were erythromycin and Sigmamycin. Sigmamycin was more active than either of its two components, oleandomycin and tetracycline. The oleandomycin-penicillin mixtures (AP and APG) were slightly more active than oleandomycin and much more active than penicillin G against these predominantly penicillin-resistant strains.

3. Tests of the bactericidal action showed erythromycin to be the most active drug in the erythromycin group. Oleandomycin was the least active of the antibiotics tested.

4. Cross-resistance between erythromycin and oleandomycin was frequent in erythromycin-resistant staphylococci but the reverse situation was not frequent.

5. Additive potentiation of the bacteriostatic activity of oleandomycin and its mixtures in paired combinations with other antibiotics (agar diffusion method) appeared most frequently with bacitracin, chloramphenicol, erythromycin, and, as expected, with other oleandomycin mixtures. Potentiation of the bactericidal activity (replica plating method) was not frequent with neomycin. Tetracycline was less effective in increasing bactericidal action than in enhancing bacteriostasis.

6. No instances of true synergism of antibacterial effects were seen with any of the antibiotic combinations studied.

7. Antagonism of antibiotic bacteriostasis was rare. Antagonism of bactericidal activity occurred more frequently, particularly with certain susceptible strains. One-third of the strains accounted for 82 per cent of the instances of antagonism.

8. Most of the antibiotic interaction, both addition and antagonism, occurred with the more susceptible strains of staphylococcus.

Technical assistance in the experiments on cross-resistance to erythromycin and oleandomycin was given by Miss Marilyn J. Johnson.

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PANCREATIC ACTION OF THE SULFONYLUREAS

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THE sulfonylurea compounds are now widely employed in the treatment of diabetes mellitus. Their exact influence on carbohydrate metabolism has not yet been elucidated. Information concerning the means by which these agents lower the blood glucose may shed light on the mechanism of pancreatic insulin secretion and the nature of diabetes itself.

Loubatières and associates[1] in 1942 began their studies of the hypoglycemic action of the isopropylthiodiazole derivative of sulfanilamide and similar drugs, and in 1946 suggested its use in diabetic patients.[2] In the last 3 years interest in this problem has revived, and a wealth of experimental data has accumulated. Many of these articles present only preliminary results or conflicting information. Stetten[3] has recently pointed out certain inconsistencies of the studies reported thus far, and called for a re-evaluation of the problem. It is the purpose of this paper to present direct evidence regarding the site of action of these drugs, and to review critically the pertinent literature.

Earlier experiments have been described elsewhere.[4-6] Acute operative studies have been performed on 59 dogs to determine the consequences of perfusing sulfonylurea solutions through the pancreas and liver. The data support the general consensus that these compounds cause hypoglycemia primarily by their influence on the pancreas. A stimulation of insulin release by the beta cells of the islets of Langerhans is postulated. The exact mode of action of the sulfonylureas may remain unknown until a better understanding is gained of the means of synthesis, storage, and secretion of insulin.

METHODS

Animals.—Small amounts of tolbutamide,* carbutamide,† or control substances were introduced into the pancreatic artery in 27, into the femoral vein in 17, into the hepatic artery in 8, and into the portal vein in 7 dogs. Healthy mongrel animals of both sexes weighing between 10 and 25 kilograms consumed a ration containing approximately 40 per cent carbohydrate, 35 per cent protein, and 25 per cent fat ad libitum for one week prior to the experiment. They were fasted for 18 hours immediately before operation.

Surgical Procedure.—Animals were anesthetized intravenously by means of 25 mg. of Nembutal for each kilogram of body weight. They were maintained in a light plane of

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†BZ-55 (or 1-butyl-3-sulfonylurea); Eli Lilly & Company, Indianapolis, Ind.

anesthesia with small subsequent doses Total amounts given over the experimental period ranged from 250 to 925 mg.

In dogs subjected to pancreatic perfusion, the right gastroepiploic artery was isolated and cannulated with a polyethylene catheter (Fig 1). The pancreaticoduodenal artery was identified at its entrance into the pancreas The test solution was injected at a slow rate in a retrograde direction by means of a constant infusion pump. In this fashion all the body and portions of the head and tail of the pancreas could be perfused without disturbing their blood supply Anatomic dissections following dye injection have indicated that approximately one third to one half of the pancreas is included by this method (Fig 2). Animals of the femoral vein group were subjected to dissection of the pancreatic circulation in order to reproduce the surgical stress obtained in the experimental dogs Pancreatic ducts were ligated in 4 animals, and 6 months later the pancreatic artery injection was performed in the manner described above

Another series of dogs received perfusion of the liver via the hepatic artery or portal vein In the former technique, the left branch of the common hepatic artery was catheterized (Fig 1) so that at least half of the organ could be perfused without completely interrupting the arterial supply Portal injection was accomplished by cannulating a splenic or mesenteric tributary.

Perfusions—Solutions containing 5 to 10 mg per milliliter of tolbutamide (sodium salt) or carbutamide were adjusted to pH 7 2. Control solutions were identical in osmolarity and pH to experimental solutions but the sulfonylurea was omitted In two animals of the pancreatic control group, sodium sulfadiazine instead of sulfonylurea was employed in the same dosage Rate of perfusion varied from 0 4 to 0 9 ml per minute and total volume from 7 0 to 19 3 ml Duration of perfusion was 20 minutes in all animals except those treated for 90 minutes in a few early screening experiments Total amount of sulfonylurea administered over these periods varied between 0 07 and 1.13 Gm Dosage of tolbutamide or carbutamide was 1 to 71 mg. per kilogram of body weight Table I illustrates that the mean dosage, volume, and perfusion rate of sulfonylurea were closely comparable for the experimental and control groups of the different injection sites

Samples—Blood specimens were withdrawn from the femoral vein immediately prior to anesthesia, operation, and perfusion and at intervals of 10 to 30 minutes following the beginning of the perfusion for 4 hours Glucose was estimated by the method of Somogyi Nelson[7] Free blood sulfonamide was determined in the carbutamide injected animals by the method of Bratton and Marshall[8] Estimate of blood concentration was not accomplished in the tolbutamide series In the carbutamide groups, biopsies were taken from the mid portion of the head of the pancreas before and after perfusion, fixed in Bouin's solution and 70 per cent alcohol, and stained with aldehyde and fuchsin to demonstrate islet cells. Frozen pancreas from some animals was assayed for insulin[9]

RESULTS

Appraisal of Experimental Procedure.—To justify comparison between the results of injection of various experimental or control solutions into the different sites, animals must be similar in physical condition, treated in the same fashion, and show a similar response to operative stress prior to perfusion. Table I gives values for these factors for groups of animals subjected to the different surgical procedures It is seen that for the different sites in both the sulfonylurea and control groups, the mean body weight (17.0 to 19 9 Kg.), amount of anesthesia (33 3 to 41 0 mg. per kilogram of body weight), and duration of surgery before perfusion (86 to 120 minutes) were comparable.

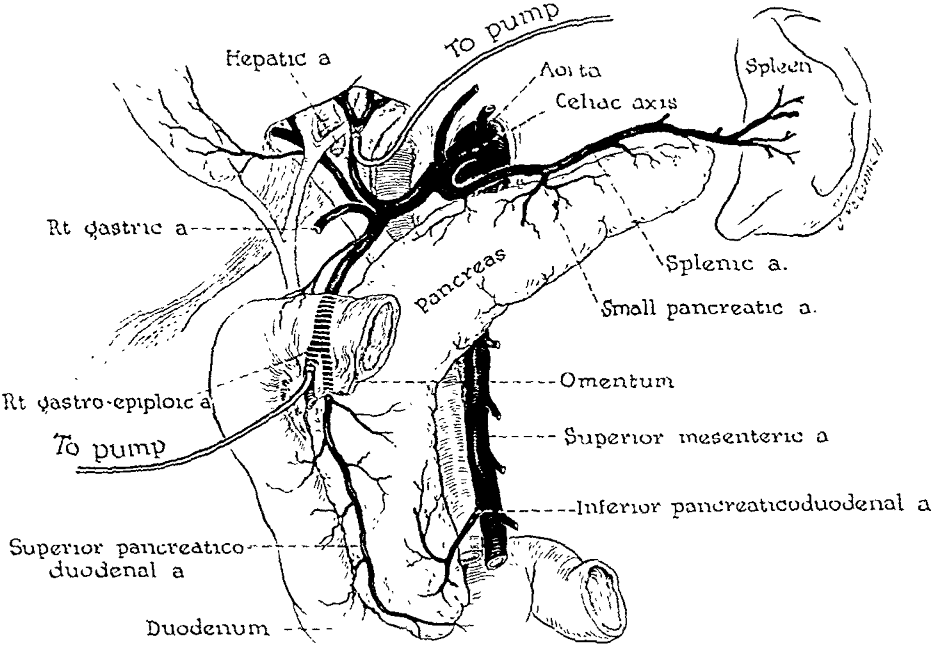

Fig. 1.—Arterial supply of the dog pancreas and liver. A polyethylene catheter has been placed in the right gastroepiploic artery so that pancreatic perfusion can be accomplished without interruption of the blood flow through the pancreaticoduodenal artery. Another catheter in the left hepatic artery permits perfusion of the liver.

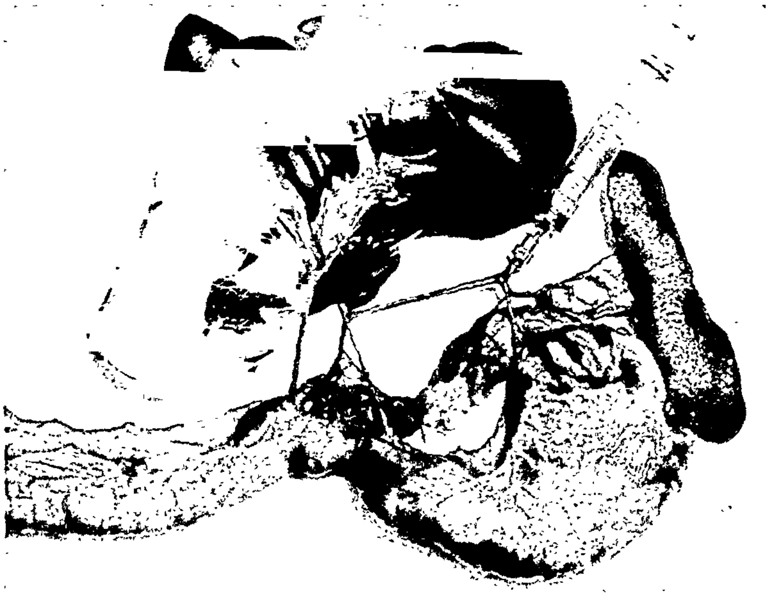

Fig. 2.—Anatomic preparation of the dog pancreas and liver, showing the arterial circulation (Fig. 1) outlined by methylene blue dye injection. An experimental solution introduced into the celiac axis is carried into the body and portions of the head and tail of the pancreas.

TABLE I. EXPERIMENTAL DATA REGARDING OPERATIVE TECHNIQUE IN EXPERIMENTAL AND CONTROL DOGS

SITE OF PERFUSION	AGENT	NO. OF DOGS	AVERAGE WEIGHT (KG.)	NEMBUTAL (MG./KG. B.W.)	AV. DURATION OF SURGERY (MIN.)	TOTAL DOSE OF SULFONYLUREA (GM.)	VOLUME (ML.)	RATE OF PERFUSION (ML./MIN.)
Pancreatic artery	Tolbutamide	6	18.6	39.3	91	0.13	12.9	0.65
	Control	4	18.6	41.0	86	--	13.0	0.65
Femoral vein	Tolbutamide	6	16.8	35.0	89	0.12	12.7	0.63
	Control	4	19.9	41.0	88	-	14.0	0.70
Hepatic artery	Tolbutamide	5	17.0	36.3	95	0.12	12.7	0.63
	Control	3	16.4	40.5	96	--	11.4	0.57
Portal vein	Carbutamide	3	16.5	35.0	132	0.12	11.6	0.55
	Control	4	14.4	33.3	114	-	10.0	0.51
Pancreatic artery (pancreatic ducts ligated)	Tolbutamide	4	17.7	41.0	120	0.12	14.2	0.71
Mean	(39 dogs)		17.3	38.0	101	(0.12)	12.6	0.62

Anesthesia caused a rise in mean blood glucose which was similar for all sites of injection.* Further increment in blood glucose during operation was almost the same for the 3 major sites.† This latter rise was also comparable in the sulfonylurea and nonsulfonylurea groups for each site (Figs. 3, 4, and 5). Dissection for cannulation of the portal vein, however, caused a 44 per cent elevation in blood glucose. Dogs in which the pancreatic ducts had previously been ligated showed 31 per cent rise in glucose with dissection of the pancreatic artery, apparently tolerating the stress of surgery poorly because of undernutrition.

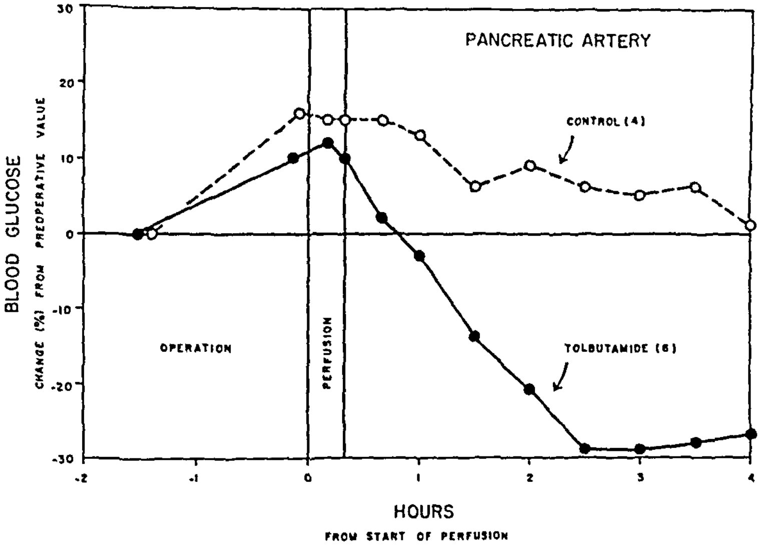

Fig. 3.—Mean curves of blood glucose following (1) pancreatic perfusion of tolbutamide in a dosage of 7 mg. per kilogram of body weight, or (2) a solution lacking sulfonylurea. The figures in parentheses refer to the number of dogs in each group.

Most of the hyperglycemia noted prior to perfusion with the different techniques was due to barbiturate anesthesia (average of all dogs, 15 per cent rise in blood glucose). There was a further increase with operation, presumably of adrenal cortical or medullary origin, which depended on the type of procedure applied. The reason for the differences in this response for the various sites is unknown. Alterations in pancreatic blood flow and disturbance of nerve pathways around the portal vein are factors which might be concerned. The fasting imposed prior to the operative procedure might have limited the capacity of the pancreas to respond to these stresses.

The question of the influence of this hyperglycemia from operation and anesthesia on the subsequent hypoglycemic response to injected agents has a direct bearing on interpretation of the data. Of course, use of control animals subjected to the same stresses should help answer this objection In addition, to determine whether anesthesia and surgery might block the peripheral effects of pancreatic insulin secretion, two animals were subjected to dissection of the pancreatic circulation under Nembutal anesthesia and were then injected

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*Average increase in blood glucose following Nembutal anesthesia (prior to surgery) in all control and experimental dogs for each site of injection was as follows: pancreatic artery 20, femoral vein 13, hepatic artery 16, portal vein 5, and pancreatic artery in conjunction with duct ligation 19 per cent. These averages were calculated from the initial level of blood glucose taken before anesthesia was given.

†Average further increase in blood glucose during the operative procedure was as follows: pancreatic artery 15, femoral vein 16, hepatic artery 6, portal vein 44, and pancreatic artery in conjunction with duct ligation 31 per cent. These averages also were calculated from the initial preanesthetic level of blood glucose and represent the further increment beyond the rise due to anesthesia (see footnote above).

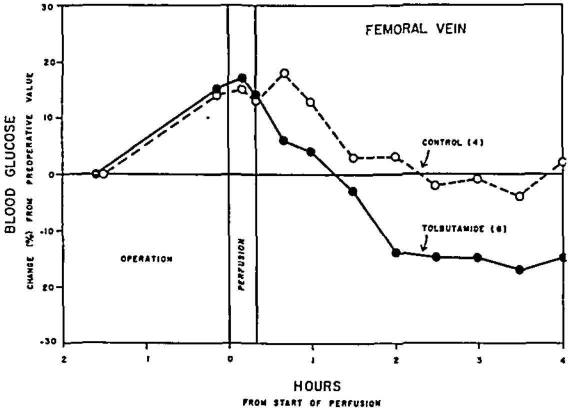

Fig 4—Mean curves of blood glucose following introduction of tolbutamide (6 dogs) or a control solution (4 dogs) into the femoral vein Dosage of tolbutamide used (7 mg per kilogram of body weight) was identical with that in the experiments depicted in Fig 3

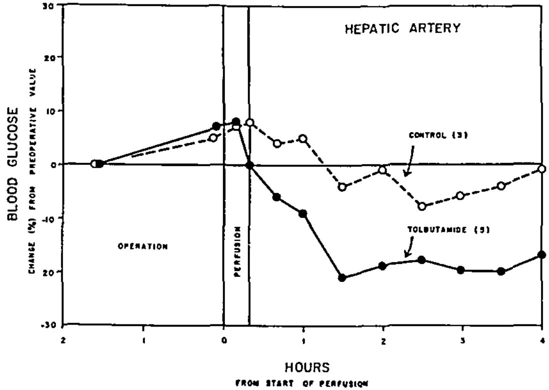

Fig 5—Mean curves of blood glucose after perfusion of tolbutamide (5 dogs) or a control solution (3 dogs) through the left branch of the common hepatic artery Dosage of tolbutamide used (7 mg per kilogram of body weight) was the same as in Figs 3 and 4

intravenously with 0.2 unit of crystalline insulin* per kilogram of body weight. An immediate drop in blood glucose occurred to a level of 50 per cent of the preinjection value, maximum at 40 minutes (Fig. 6). The same amount of insulin given to two unanesthetized, unoperated animals produced only a slightly greater and more sustained hypoglycemia. This indicates that a response to insulin can be demonstrated under the conditions of the surgical technique employed, despite the complicating factors mentioned above.

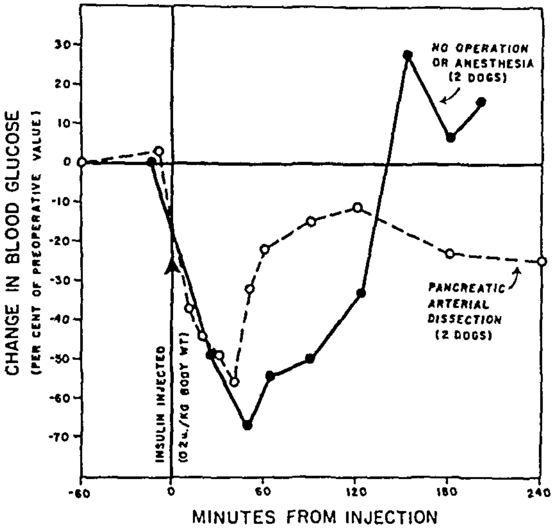

Fig. 6.—Mean fall in blood glucose from intravenous injection of crystalline insulin in a dosage of 0.2 unit per kilogram of body weight. In 2 anesthetized animals, the pancreatic artery had been isolated. Two other animals were not subjected to surgery.

Following perfusion the control (nonsulfonylurea) animals exhibited only minor differences in response depending on the site of injection utilized. Blood glucose returned to its preoperative level promptly in animals of the femoral vein and hepatic artery groups (Figs. 4 and 5), whereas it remained somewhat elevated in the pancreatic artery dogs (Fig. 3). It is possible that the arterial perfusion technique somehow reduced slightly the insulin output of the pancreas. There was no correlation in the individual animals of any group between the degree of preinjection rise and of postinjection fall in blood glucose.

A definite drop in blood glucose from the preoperative levels failed to occur with injection of the control solution into the various blood vessels. Therefore, it may be said that the hypoglycemia in the experimental animals was a result of the sulfonylurea and that the manipulative procedure did not contribute to it. Comparison of the mean reduction of blood sugar in sulfonamide-injected animals with the curves obtained on their own controls seems more valid than comparison of results of perfusion at the various sites.

Pancreatic Artery.—Pilot experiments were performed initially to establish the blood glucose response to injection of carbutamide into the pancreatic artery. Dosage ranged between 12 and 71 mg. per kilogram of body weight.

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*Iletin, manufactured by Eli Lilly & Company, Indianapolis, Ind.

Table II shows that the maximum hypoglycemia produced by the drug (2 to 3 hours after perfusion) varied roughly with the dosage and the resulting blood sulfonylurea level. The amount of carbutamide was progressively reduced in order to obtain the minimum concentration which would still produce hypoglycemia, presumably by a local pancreatic effect. When a dosage of 7 mg. per kilogram of body weight was used, producing a venous blood sulfonylurea level (2 to 4 mg. per cent) too low for therapeutic effect, a fall in blood glucose occurred. If the same amount of carbutamide was injected into the femoral vein instead of the pancreatic artery, no such hypoglycemia was noted.[4] With the exception of the portal vein perfusions, all subsequent experiments utilized the same small dose (7 mg. per kilogram) of tolbutamide.

Fig. 3 illustrates the mean curves of change in venous blood glucose following perfusion of the pancreatic artery with tolbutamide or an identical solution lacking tolbutamide. Raw data for the individual experiments are given in Table III. Mean blood sugar dropped approximately 30 per cent from the preoperative value in the 6 experimental animals. The effect is somewhat greater if the control (nonsulfonylurea) curve is used for a base line of reference. Hypoglycemia failed to occur in the 4 control dogs. Both groups showed a rise in blood glucose with the stress of surgery and anesthesia. Although slightly greater in the control animals, this was generally comparable in the two groups.

Femoral Vein.—For the purpose of comparison with the results of pancreatic perfusion, 10 animals were subjected to sham operation on the arterial circulation of the pancreas and then tolbutamide or a control solution was injected into a femoral vein. Results of these studies are given individually in Table III, and mean curves are shown graphically in Fig. 4. Maximum drop in blood glucose was somewhat greater in the experimental dogs (15 per cent) than in the control group (4 per cent). However, the difference between the two curves is only half that between the experimental and control curves following pancreatic perfusion (Fig. 3). Making use of the control data, analyses showed a significant difference between the responses for the femoral vein and pancreatic artery groups.*

Hepatic Artery.—The same dose (7 mg. per kilogram of body weight) of tolbutamide (5 dogs) or a control solution (3 dogs) was perfused into the left hepatic artery. Hypoglycemia of 20 per cent occurred in the sulfonylurea injected animals (Fig. 5), approximately the same response seen in the femoral vein group. Control dogs in which the hepatic artery was perfused with a solution lacking sulfonylurea exhibited little or no hypoglycemia. The difference between the control and experimental curves was much smaller than the response to injection of the pancreatic artery (Fig. 3). The changes following perfusion were found to be significantly different when the hepatic artery and pancreatic artery data were compared.*

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*Statistical analyses utilizing mean changes from the preoperative blood glucose levels demonstrated a significant difference between values for the pancreatic artery and femoral vein groups ($p<0.01$) also between values for the pancreatic artery and hepatic artery groups ($p<0.01$). There was no difference between values for the femoral vein and hepatic artery groups.

Table II. Results of Perfusion of Carbutamide Into the Pancreatic Artery

DOSE (MG./KG.)	MAXIMUM FALL IN BLOOD GLUCOSE (PER CENT)	MAXIMUM FREE VENOUS BLOOD SULFONAMIDE (MG. PER CENT)
71	–49	15.7
57	–36	--
16	–45	6.7
12	–28	4.1
7	–12	3.0

Table III. Individual Blood Glucose Values in Dogs Before and After Perfusion
Experimental Solutions

DOG NO.	PERFUSION SITE	AGENT	BLOOD GLUCOSE (MG. PER CENT) MINUTES BEFORE OR AFTER BEGINNING OF PERFUSI										
			–90	–10	10	20	40	60	90	120	150	180	210
88	Pancreatic artery	Tolbutamide	88	91	92	89	92	81	61	54	44	46	47
89	Pancreatic artery	Tolbutamide	85	88	91	89	71	72	69	71	73	68	65
107	Pancreatic artery	Tolbutamide	93	112	114	112	110	106	87	72	50	59	68
115	Pancreatic artery	Tolbutamide	82	98	103	104	101	96	88	83	73	71	78
117	Pancreatic artery	Tolbutamide	88	98	94	89	79	74	76	70	72	69	66
118	Pancreatic artery	Tolbutamide	85	86	89	90	78	75	68	59	57	56	51
87	Pancreatic artery	Saline	82	96	--	90	96	97	96	98	100	93	100
90	Pancreatic artery	Saline	72	95	93	98	92	85	80	85	79	72	78
120	Pancreatic artery	Sulfadiazine	75	82	81	78	81	82	70	75	69	74	74
121	Pancreatic artery	Sulfadiazine	78	84	85	85	83	84	80	84	84	84	74
98	Femoral vein	Tolbutamide	92	111	122	121	115	112	102	84	66	62	58
105	Femoral vein	Tolbutamide	76	91	93	94	87	94	85	88	86	86	81
106	Femoral vein	Tolbutamide	80	85	88	85	83	76	72	68	67	57	61
114	Femoral vein	Tolbutamide	--	81	84	80	74	75	62	43	60	54	58
116	Femoral vein	Tolbutamide	71	83	83	79	73	65	67	61	68	69	69
119	Femoral vein	Tolbutamide	86	90	86	81	72	70	73	70	70	72	61
129	Femoral vein	Saline	73	92	98	97	102	91	80	78	72	72	69
130	Femoral vein	Saline	84	104	103	101	100	96	88	88	88	92	89
131	Femoral vein	Saline	93	96	93	91	93	96	86	90	85	84	80
132	Femoral vein	Saline	87	85	86	86	90	90	90	88	86	84	86
108	Hepatic artery	Tolbutamide	92	110	110	101	97	100	82	81	93	86	85
109	Hepatic artery	Tolbutamide	94	88	89	83	74	80	71	70	72	67	70
110	Hepatic artery	Tolbutamide	73	75	76	70	72	65	50	61	57	65	54
122	Hepatic artery	Tolbutamide	72	85	84	82	79	63	58	60	54	45	58
123	Hepatic artery	Tolbutamide	92	92	90	86	72	75	75	71	72	79	74
111	Hepatic artery	Saline	94	96	93	93	86	93	82	83	80	79	84
112	Hepatic artery	Saline	100	106	111	111	114	105	99	100	86	90	92
125	Hepatic artery	Saline	71	77	78	79	76	78	73	78	74	76	77
85	Portal vein	Carbutamide	78	125	130	133	117	139	148	136	124	116	117
91	Portal vein	Carbutamide	73	130	124	116	127	124	121	93	77	64	56
92	Portal vein	Carbutamide	96	118	144	138	134	134	128	110	102	98	94
86	Portal vein	Saline	86	130	150	152	154	156	156	158	128	114	102
133	Portal vein	Saline	85	85	85	85	86	83	85	80	80	78	71
136	Portal vein	Saline	78	96	88	88	79	93	78	74	78	69	78
137	Portal vein	Saline	83	130	130	123	111	105	89	83	75	75	79
124	Pancreatic artery	Tolbutamide	85	98	102	99	96	87	65	67	69	77	82
126	(ducts ligated)	Tolbutamide	86	114	113	110	105	103	98	77	85	86	80
127	Pancreatic artery	Tolbutamide	83	91	85	94	87	93	81	68	68	65	67
128	(ducts ligated)	Tolbutamide	92	139	138	137	128	142	123	113	96	96	94

Comparison of Responses—In order to take into account the hyperglycemia from anesthesia, the experimental curves have been redrawn in Fig 7, using the preanesthetic blood glucose as the basal reference point It is noted that *subjecting the pancreas to the sulfonylurea solution caused a definite hypoglycemia*, whereas the other techniques produced little or no reduction in blood sugar below the preanesthetic level Thus, no matter whether the response to perfusion of the experimental agents is compared to the blood glucose taken *before* or *after* anesthesia (which did cause a rise in glucose concentration averaging 15 per cent), the differences between the results of injection at the various sites are maintained *

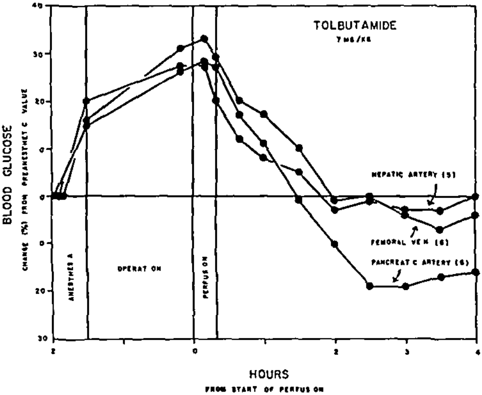

Fig 7—Mean change in blood glucose calculated from the preanesthetic (instead of the preoperative) value for the 3 major sites of perfusion

Portal Vein—Marked hyperglycemia, two or three times as great as that observed with other operative procedures, occurred when a tributary of the portal vein was cannulated (Fig 8) Unfortunately, this effect was somewhat less in the control (saline) than in the experimental (sulfonylurea) animals, and accurate comparison is therefore impossible However, injection of carbutamide (7 mg per kilogram of body weight) into the portal vein failed to produce hypoglycemia

Pancreatic Artery With Ligation of the Pancreatic Ducts—The excretory ducts of the pancreas were ligated in 4 animals Six months later, the pancreatic artery was perfused with tolbutamide (7 mg per kilogram) by the usual method Again, preoperative rise in blood glucose was excessive (Fig 9), but hypoglycemia was obtained However, since control data are not available, no conclusions can be drawn

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*See footnote on page 383

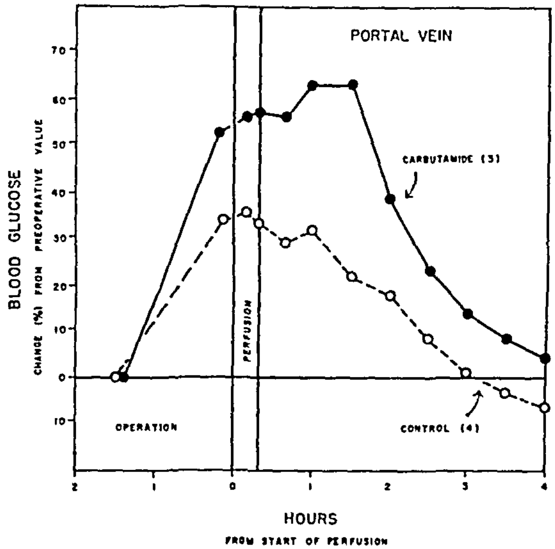

Fig 8.—Mean curves of blood glucose after injection of carbutamide (3 dogs) or a control solution (4 dogs) into a vessel entering the portal vein Dosage of sulfonylurea used (7 mg per kilogram of body weight) was the same as in Figs. 3, 4, and 5.

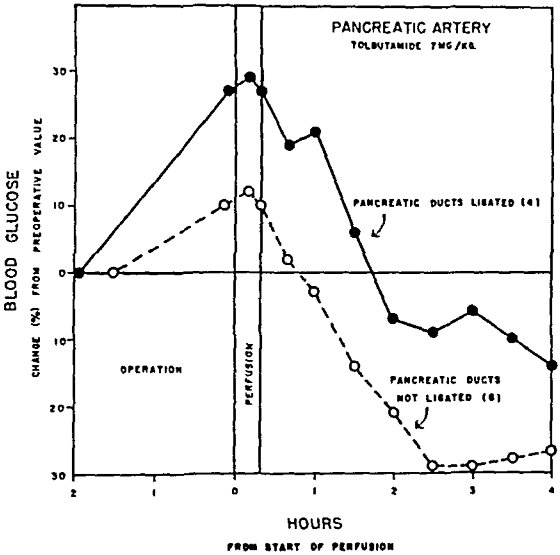

Fig 9.—Mean curves of blood glucose after injection of tolbutamide into the pancreatic artery in animals with (4 dogs) or without (6 dogs) prior ligation of the pancreatic ducts Dosage of tolbutamide used (7 mg per kilogram of body weight) was the same as in Figs 3, 4, 5, and 6

Pancreatic Insulin Assay and Biopsies.—Results of analyses of insulin content of frozen pancreas taken from some animals are given in Table IV. Since not all specimens were assayed, these data are incomplete. Definite differences between the various experimental groups were not obtained. Pancreatic biopsies obtained from experimental and control animals injected with carbutamide failed to show alteration in morphology of alpha or beta cells.

TABLE IV. MEAN INSULIN CONTENT OF PANCREAS FOLLOWING PERFUSION OF EXPERIMENTAL AND CONTROL SOLUTIONS

NO. OF DOGS	AGENT	DOSE (MG./KG. OF B.W.)	PANCREATIC INSULIN (μ/GM.)
		Pancreatic Artery	
2	Tolbutamide	7	1.2
2	Tolbutamide	3	1.8
2	Tolbutamide	1	2.6
2	Carbutamide	7	2.7
1	Saline		1.9
		Femoral Vein	
3	Tolbutamide	7	2.1
2	Tolbutamide	3	2.0
4	Carbutamide	7	2.2
		Portal Vein	
3	Carbutamide	7	1.4
1	Saline	–	0.9

DISCUSSION

It was the intention of these experiments to determine the behavior of the venous blood glucose on exposure of certain organs to a low concentration of sulfonylurea. It was hoped that the site of action of these agents could thus be localized. First, moderate amounts (12 to 71 mg. per kilogram of body weight) of carbutamide were injected into the pancreatic artery. A minimal dosage (7 mg. per kilogram of body weight) was established which would produce peripheral hypoglycemia when introduced into the pancreas but not when introduced into the femoral vein. Then the same amount of tolbutamide was perfused through the pancreas or liver by way of the appropriate blood vessels. Hypoglycemic response from perfusion of the pancreatic artery was significantly greater than that from perfusion of the femoral vein or hepatic artery. This difference was more pronounced if the degree of drop in blood glucose for each site of injection was judged from its own control curve (where a solution lacking sulfonylurea was employed). No hypoglycemia was obtained from perfusion of these control solutions. The dissimilarity between the responses was borne out further when the alteration in blood glucose was calculated from the preanesthetic instead of from the postanesthetic level.

The results show that maintaining a low concentration of tolbutamide in the pancreatic circulation for a period of 20 minutes influenced the organ in some way to lower the peripheral venous blood glucose. With the dosage employed, an average of 6 mg. of tolbutamide was added to the pancreatic circulation each minute for 20 minutes. The rate of blood flow through the pancreatic artery in the experiments is unknown. If this were postulated to be 10 ml. per minute, then a therapeutic concentration (10 mg. per cent) of sulfonylurea in the peripheral blood would supply the pancreas with approximately 1 mg. per minute. Although the sulfonylurea content of the pancreatic venous blood was not measured, it may be assumed that it was considerably greater than that of the peripheral circulation. The maximum venous free sulfonamide level in animals injected with carbutamide (7 mg. per kilogram) was only 3.0 mg. per cent (Table II), well below the effective therapeutic level. Concentrations of tolbutamide in the pancreatic and peripheral blood were undoubtedly comparable to those of carbutamide since the same dosage was administered. Injecting the same amount of the agent into the femoral vein or hepatic artery failed to produce a similar effect on the blood glucose.

Curves obtained from perfusion of the arterial circulation to the duct-ligated pancreas were inconclusive. Destruction of the acinar tissue did not seem to influence the usual hypoglycemic response to tolbutamide injection. The opportunity to subject similarly prepared animals to perfusion of solution without sulfonylurea was lacking. Evidence that the acini are involved in the pancreatic action of the sulfonylureas has never been presented. It is felt that the suboptimal nutritional state of the dogs, manifested by an increased hyperglycemic response to stress, may have impaired the usual functional response of the pancreas.

Results obtained from estimation of pancreatic insulin content failed to establish any consistent differences between the values for the various sites of perfusion (Table IV). However, it is interesting and suggestive that the mean values following pancreatic injection bore a rough relation to the amount of tolbutamide used. Larger doses of the drug seemed to cause a greater fall in insulin concentration. These data, although incomplete, are compatible with the idea that the sulfonylurea compounds lower the blood glucose by stimulating a release of insulin from the pancreas.

The technique of arterial perfusion described proved valuable in investigating pancreatic islet function. Test materials other than the sulfonylureas might be injected in a similar manner to assess their influence on the islet cells.

Liver Perfusion.—A marked hyperglycemia was obtained prior to perfusion with carbutamide of the portal circulation via one of its tributaries. There was no sign of a peripheral hypoglycemia from exposing the liver to the carbutamide. Moreover, perfusion of tolbutamide (7 mg. per kilogram) into the left hepatic artery caused little or no fall in blood glucose from the preoperative level (Fig. 5). These results constitute strong evidence against an hepatic action as the primary reason for the peripheral hypoglycemia caused by the sulfonylureas. They also indicate that the drugs do not act on the liver to cause hypoglycemia after leaving the pancreas. The liver may have a role in the blood glucose alteration from these agents, but this function is probably secondary to insulin release by the pancreas.

REVIEW OF THE LITERATURE

A critical evaluation of the evidence in the literature regarding clinical and animal experiments with the sulfonylureas bears out the hypothesis of a pancreatic site of action. The presence of endogenous insulin is necessary for the hypoglycemic effect. Influences on other organs, notably the liver and thyroid gland, are also seen, particularly after high doses. Hepatic effects are complicated and may be dependent on pancreatic function. No organ except the pancreas is essential for the hypoglycemic response. The agents have no influence on blood glucose in the eviscerated animal.[10] It is our intention to examine briefly the important data which support these general statements. Table V summarizes salient findings by workers interested in this problem.

Adrenal.—Neither the adrenal cortex nor the adrenal medulla needs to be available for the sulfonylureas to be effective.[11-13] Total adrenalectomy potentiated the fall in blood glucose following the administration of the compounds; this was not true after isolated removal of either cortex or medulla. Steroid excretion in the urine was not altered by carbutamide or tolbutamide therapy in human beings.[14-16]

Pituitary.—Following hypophysectomy, there was a normal response to sulfonylurea administration.[11, 17, 2]

Thyroid.—Although depression of thyroid activity as measured by the radioactive iodine uptake and blood protein bound iodine level has been described after large doses,[18] ordinary therapeutic amounts had no such effect.[9]

Liver.—Presence of the liver is not essential to obtain a blood glucose response from the sulfonylureas.[20] Glucose output from the liver is reduced,[21-23] but attempts to link this phenomenon with inhibition of specific enzyme systems have been fruitless. The glycolytic enzymes phosphorylase and glucose 6 phosphatase have been studied extensively, and the consensus is that neither is significantly affected with the doses commonly used.[24-26] Glucagon and epinephrine hyperglycemia, presumably mediated through the phosphorylase system, is not altered by the sulfonylureas.[27, 28] Changes in liver glycogen are variable depending on the experimental procedure and dosage used.[9, 29, 30] Mirsky's early observations that "insulinase" is inhibited[31] have been contradicted by others employing smaller amounts of the drugs.[32, 33] The rate of degradation of insulin tagged with I^{131} is not altered.[32] Although the sulfonamides may antagonize certain inhibitors of insulin, there is no proof that this is related to their hypoglycemic effect.

The major action of the sulfonylureas on the liver appears to be the depression of glucose output. Whether this is primary or secondary to insulin stimulation has not been determined. High concentrations of the drugs may produce nonspecific enzyme inhibitory effects, some of which may be responsible for reducing hepatic glucose manufacture. None of these has been demonstrated to prevail at reasonable dosages or to account for the fall in peripheral blood glucose.

artery in the experiments is unknown. If this were postulated to be 10 ml. per minute, then a therapeutic concentration (10 mg. per cent) of sulfonylurea in the peripheral blood would supply the pancreas with approximately 1 mg. per minute. Although the sulfonylurea content of the pancreatic venous blood was not measured, it may be assumed that it was considerably greater than that of the peripheral circulation. The maximum venous free sulfonamide level in animals injected with carbutamide (7 mg. per kilogram) was only 3.0 mg. per cent (Table II), well below the effective therapeutic level. Concentrations of tolbutamide in the pancreatic and peripheral blood were undoubtedly comparable to those of carbutamide since the same dosage was administered. Injecting the same amount of the agent into the femoral vein or hepatic artery failed to produce a similar effect on the blood glucose.

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necrosis, hypoprothrombinemia, and hemorrhage have been described in depancreatized dogs.[36, 37] This may be related to the nutritional deficiencies that occur when there is loss of the digestive functions of the pancreas.

Pancreas.—The sulfonylureas are ineffective in totally depancreatized animals.[11, 17] Hypoglycemic response to the compounds is normal in partially pancreatectomized man.[38] That the alpha cells of the islets or their function of elaborating glucagon are not involved is shown by the lack of effect in alloxan diabetes,[1, 39] the absence of any morphologic damage to the alpha cells after oral administration,[28, 40] and the failure of the sulfonamides to influence the hyperglycemic response to injected glucagon.[27, 28]

Functioning beta cells must be available for a response to occur. There is a high correlation between the amount of insulin extractable from the human pancreas and the occurrence of a decline in blood glucose as a result of administration of the sulfonylureas.[41] Patients with juvenile diabetes, known to exhibit a low pancreatic insulin content, are largely unresponsive except early in the course of their disease.[42] Ashworth and Haist's[43] observation that an increase in islet volume and weight occurs after sulfonylurea administration is consistent with the hypothesis of a stimulus to insulin secretion. Experiments on cross circulation also support this concept.[44] Degranulation of beta cells has been observed after prolonged treatment,[28] but whether this represents insulin stimulation or functional rest is difficult to decide. Toxic doses cause a reduction in pancreatic insulin content in the experimental animal, but therapeutic doses do not.[9] Von Holt and associates[45] have demonstrated a rise in plasma insulin in acute experiments on rats. Goetz and Egdahl[46] recently found that tolbutamide produced an increase in insulin output into the pancreatic vein. To date, there is no convincing evidence that these compounds cause an "exhaustion" of pancreatic islet cells by means of repeated stimulation of insulin release.

Despite the evidence cited favoring an action of the sulfonylureas involving insulin synthesis or release, the usual metabolic effects of injected insulin are strangely lacking. That the normal patient develops a striking hypoglycemia and the diabetic a lesser response is agreed. Two well controlled studies show a decrease in blood potassium and phosphorus similar to that following insulin.[47, 48] The depression of phosphorus after insulin injection implies entry of glucose in the cell; yet, Wick and co-workers[49] were unable to show in eviscerated animals that the sulfonylureas cause an increased intracellular transfer of glucose. There are, also, other contradictory data. Goetz and associates[48] and Madison and Unger[50] found that the sulfonylureas cause an increased arteriovenous glucose difference, again implying cellular entry of glucose. Purnell and co-workers,[22] with equally well controlled experiments, were unable to demonstrate such an effect. Muscle and liver glycogen data are particularly confusing, with results reported which are diametrically opposed.[9, 12, 13, 30, 51] Undoubtedly variations in technique play a large role in the glycogen evidence. Dulin and Johnston[20] found that they could produce hypoglycemia by means of small intravenous doses of insulin without alteration in liver glycogen, an effect comparable to that of tolbutamide. Tolbutamide was found to lower blood ketones and plasma unesterified fatty acids in diabetic patients.[52] However, evidence regarding fat metabolism is largely inadequate. Hennes and associates[53] described a fall in blood pyruvate immediately following tolbutamide administration, in contrast to the early rise after insulin. Using insulin doses calculated to produce the same hypoglycemic effect as the sulfonylureas, Miller and his co-workers[23] were unable to demonstrate any difference in pyruvate response. If the agents stimulate insulin release, one might expect an increased oxidation of glucose by the peripheral tissues. Miller and associates[54] were able to show this by carbon labeling, but Wick and associates[49] were unable to do so. Insulin effects which could *not* be demonstrated employing the sulfonylureas include correction of negative nitrogen balance,[11] increase of respiratory quotient,[55] improvement in glucose tolerance,[27] and increased glucose uptake by muscle[26, 29, 56] and adipose tissue.[57] Certainly, the compounds are not truly antidiabetic since they fail to correct the metabolic abnormalities resulting from insulin deficiency. Indeed, this fact mitigates against our hypothesis that they cause hypoglycemia by releasing pancreatic insulin.

The data presented in this article, as well as the evidence cited, favor a primary influence of the sulfonylurea compounds on the pancreas. Introducing a very small amount of tolbutamide (average, 6 mg. per minute for 20 minutes) into the pancreas provoked a hypoglycemic effect, whereas injection of the same dose into the liver or a peripheral vein did not. Urgoiti in Houssay's laboratory injected larger dosages of tolbutamide into the pancreatic artery by a different technique and was unable to demonstrate definitely a stimulating effect.[58] However, a greater drop in blood glucose was found with pancreatic artery injection than with saphenous or portal vein injection (not statistically significant). Loubatières[59] produced hypoglycemia by injection of the isopropylthiodiazole derivative of sulfanilamide (40 mg. per kilogram of body weight) into the pancreatic artery or pancreatic duct.

It is our view that tolbutamide or carbutamide in the pancreatic arterial blood cause insulin to be released in a slow, sustained fashion from the beta cells of the islets into the pancreatic vein so that there is a reduction in hepatic and therefore peripheral venous blood glucose. Some of the contradictions in the experiments mentioned might be explained by the timing, dosages, and species differences involved. It may be impossible to demonstrate the peripheral effects of insulin on tissue or muscle by present techniques if small amounts are released slowly by the pancreas.

SUMMARY

Site of action of the sulfonylureas was investigated by injecting solutions of the compounds into the blood vessels supplying the pancreas and liver. Perfusion of the pancreas via its arterial circulation with a small amount of tolbutamide in the anesthetized dog caused moderate hypoglycemia. Little or no such effect was obtained when the femoral vein or hepatic artery was perfused with the same amount (7 mg. per kilogram of body weight) of the drug. Mean response of the pancreatic artery group was significantly different from

those of the femoral vein or hepatic artery groups The primary importance of the liver in the response was further refuted by a lack of hypoglycemia following injection of carbutamide into the portal vein Although ligation of the pancreatic ducts failed to prevent the hypoglycemic response to sulfonylurea introduced into the pancreatic circulation, no conclusions may be drawn regarding an acinar role because of absence of control information There was no alteration in morphology of alpha or beta cells following perfusion of the main pancreatic artery with carbutamide

The important literature bearing on the question of the mechanism of action of these compounds is reviewed, and the nature of their influence on the pancreas is discussed The data support the idea that the sulfonylurea compounds cause a decrease in blood glucose by virtue of their effect on the pancreatic islets These experiments tend to establish the usefulness of the technique of pancreatic perfusion for estimating the effect of various substances on islet function

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THE EFFECT OF PREDNISONE AND AMPHENONE ON FLUID AND ELECTROLYTE BALANCE AND ON ALDOSTERONE EXCRETION OF PATIENTS WITH CIRRHOSIS AND ASCITES

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THE accumulation of ascites and edema by patients with chronic liver disease is associated with a low sodium content of urine, sweat, and feces,[1-4] and adrenal hormones have been held responsible in part for this phenomenon. Recent observations have given added support to this hypothesis. Aldosterone, a potent salt-retaining hormone isolated from adrenal venous blood,[5] is excreted in excessive amounts by patients with cirrhosis and ascites.[6-8] Moreover, bilateral adrenalectomy has been performed for treatment of rapidly accumulating ascites in human beings with cirrhosis,[9, 10] although this seems a formidable procedure when rigid sodium restriction alone will halt further ascites formation.[1, 2, 11]

Amphenone†[12, 13] inhibits adrenal cortical steroid secretion in animals and man.[14-18] The clinical usefulness of this compound is hampered by its toxic effects (i.e., skin rash, drowsiness, gastrointestinal symptoms, methemoglobinemia, and impaired liver function tests). However, Renold and associates[19] have found only mild toxic manifestations in man when Amphenone was given for only a short time.

To help elucidate the role of adrenal cortical steroids in the pathogenesis of sodium retention as observed in cirrhosis with ascites, Amphenone was given to inhibit steroid production. The untoward side effects of this drug and the potential hazard of adrenal insufficiency necessitated the coadministration of prednisone which provided the opportunity to evaluate its diuretic properties. Thus, prednisone and Amphenone were administered to a selected group of patients resistant to standard diuretic therapy. The effect of these drugs on fluid balance and on urine sodium and aldosterone excretion was studied.

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†Amphenone, 3,3 [illegible] has been generously supplied by The Upjohn [illegible] supplied through the courtesy of Merck Sharp [illegible] Rahway, N J

MATERIALS AND METHODS

Ten patients with documented cirrhosis complicated by ascites and edema were selected for study. The group comprised of 7 men and 3 women, aged 40 to 67 years, who were resistant to diuretic therapy with salt restriction and mercurials. Diagnosis was established by history, physical examination, and laboratory studies in each case. In 7 instances, confirmatory histologic evidence was also available. Nine patients gave a history of chronic alcoholism and poor diet for from 6 to 40 years, and the remaining patient (Case 7) had cirrhosis of undetermined etiology confirmed by liver biopsy. A history of previous jaundice, which seemed related to acute hepatic decompensation of the alcoholic, was elicited in 2 patients. Ascites had been present continuously for from 2 to 18 months prior to study, although 7 patients had had persistent ascites of only 3 months' duration or less. Three patients had previously had paracenteses and 3 gave a history of intermittent ascites over a 1- to 6-year period. All patients showed evidence of poor nutrition; severe wasting was present in 6, while 2 were obese. Ascites was severe in all cases. Case 10 showed signs of mild cardiorespiratory insufficiency which was relieved as ascites improved. Accurate estimation of liver and spleen size was prevented by ascites. X-ray examination of the esophagus did not reveal varices in any patient; however, 7 had enlarged abdominal collateral veins and 4 had hemorrhoids. A history of recent impending hepatic coma was present in 3 cases (Cases 4, 8, and 10), and Case 1 had arteriosclerotic heart disease with atrial fibrillation, which was well stabilized with maintenance digitalis throughout the study.

All patients were ambulatory on the Thorndike metabolic ward and were maintained on a constant diet supplying 10 mEq. or less of sodium, approximately 100 mEq. of potassium, 50 to 80 Gm. of protein, and at least 2,200 calories daily as calculated from standard tables.[20] Direct analysis of a daily diet was performed on 2 occasions revealing 4.6 and 4.9 mEq. of sodium, 109 and 100 mEq. of potassium, and 9.5 and 11.3 Gm. of nitrogen for Cases 9 and 10, respectively. Fluids were allowed ad libitum, except for Cases 5 and 6 who were restricted to 1,600 ml. and 2,000 ml. per day, respectively. The studies were made during cool weather in all but 3 patients (Cases 1, 2, and 3), minimizing variations in skin losses of sodium and water. Fecal and sweat losses of sodium were assumed to be 8 to 10 mEq. per day[11] and the remaining insensible fluid loss to be 800 to 1,000 ml. per day. No patient experienced diarrhea or vomiting during the study.

The patients were weighed at the same time each day. Daily fluid intake was recorded. Total 24 hour urine output was measured and daily aliquots taken for determination of sodium, potassium, chloride, and nitrogen. In selected cases samples were saved for aldosterone determination, and a sample was frozen for osmolality measurements. Blood specimens were obtained from an antecubital or femoral vein without stasis. Determinations of hematocrit, serum potassium, sodium, chloride, albumin, nonprotein nitrogen, and bilirubin were made before each change in therapy and at other indicated times. Daily clinical evaluations were made and recorded.

Methods used in this laboratory for the above determinations have been previously recorded,[21, 22] with the exception of serum albumin, which was measured by the method of Rutstein and associates.[23] Total solute concentration in urine and serum was measured by freezing point depression using a Fiske osmometer, and results are recorded as osmolal concentrations.

Urinary aldosterone excretion was measured by a modification[24] of the Neher and Wettstein method.[25] Unconjugated aldosterone is known to be easily destroyed when exposed to strongly acid conditions.[26] Thus, in view of the possibility that impaired liver function might result in defective conjugation of aldosterone, all but 3 urine specimens were extracted twice: (1) within one hour after acidification to pH 1 (this fraction is designated "free" aldosterone), and (2) again 48 hours later (this fraction is designated "conjugated" aldosterone). The combined extraction, performed on 6 urines of patients

TABLE I. METABOLIC BALANCE STUDIES IN 10 PATIENTS WITH CIRRHOSIS AND ASCITES

PATIENT, AGE, SEX, WEIGHT AND DATE	DAY OF STUDY	PERIOD OF STUDY*†	MEAN WEIGHT CHANGE PER DAY (KG.)	MEAN 24 HR. URINE					MEAN 24 HR. FLUID INTAKE (ML.)
				SODIUM (MEQ.)	POTASSIUM (MEQ.)	CHLORIDE (MEQ.)	NITROGEN (GM.)	VOLUME (ML.)	
1. 62, M	1-6	I	-0.1	2	58	28	---	1,173	---
79.6 Kg.	7-9	II	-0.06	5	74	24	---	1,536	---
8/18/56	10-12	III	+0.2	13	58	51	---	944	---
	13-15	IV	-0.46	49	116	101	---	1,830	---
	16-17	V	+0.3	2	30	35	---	1,180	---
2. 40, M	1-6	I	-0.26	5	28	24	---	896	---
105 Kg.	7-9	II	-0.33	1	50	7	---	1,326	---
8/11/56	10-12	III	-0.33	1	69	14	---	2,298	---
	13-16	IV	-0.30	1	78	19	---	2,402	---
	17-18	V	-0.55	44	101	8	---	1,670	---
	26-32	IIa	-0.2	1	68	24	---	1,981	---
3. 67, M	1-6	I	+0.28	1	30	6	---	830	2,116
72 Kg.	7	A	-0.5	1	32	2	---	390	2,135
9/6/56	8-10	II	-0.9	7	77	16	---	1,360	1,758
	11-14	III	-0.25	25	84	57	---	991	2,000
	15-18	IV	-0.5	12	48	34	---	681	1,880
	19-25	V	+0.29	10	36	--	---	587	1,670
	26-30	IIa	-0.18	1	23	3	---	765	1,877
	31-35	Va	-0.1	1	36	2	---	617	1,851
4. 42, F	1-7	I	+0.01	1	18	3	---	418	1,444
47 Kg.	8-15	II	+0.13	1	24	5	6.1	899	1,686
10/11/56	16-21	V	-0.12	55	54	30	4.8	962	1,949
	22-25	Vb	+0.17	1	30	2	5.0	735	1,615
5. 46, M	1-7	I	-0.05	1	20	4	3.4	516	1,648
62 Kg.	8-16	II	-0.41	33	35	33	4.8	971	1,596
10/31/56	17-22	V	-0.29	45	36	38	3.1	840	1,600
6. 42, F	1-5	I	-0.00	12	63	16	3.8	1,445	1,985
64 Kg.	6-17	II	-0.42	61	71	67	5.4	2,069	2,091
1/14/57	18-26	V	-0.23	44	57	52	---	2,141	2,000
7. 63, F	1-7	I	+0.26	2	39	10	4.6	1,115	2,214
64 Kg.	8-17	II	-0.02	14	40	19	4.5	1,566	2,704
1/26/57	18-20	III	-0.17	35	41	50	5.1	1,540	2,813
	21-26	IV	+0.05	18	44	47	6.4	1,512	2,650
	27-37	IVc	+0.06	3	43	12	5.3	1,400	2,644
	38-42	V	+0.16	1	38	38	---	966	2,640
8. 42, M	1-6	I	+0.03	7	28	45	---	988	2,080
63 Kg.	7-14	II	-0.01	1	35	18	---	1,384	2,105
2/7/57	15-18	III	-0.25	29	56	38	---	1,490	2,485
	19-20	IV	-0.7	36	61	47	---	1,030	1,870
9. 40, M	1-6	I	+0.36	2	54	24	4.9	783	1,555
81 Kg.	7-17	II	-0.64	68	111	101	7.7	1,966	1,719
3/1/57	18-23	III	-0.81	67	114	129	7.0	2,001	2,001
	24-30	IV	+0.05	4	96	26	8.9	1,346	1,825
	31-36	V	+0.07	1	89	22	8.7	859	1,441
10. 42, M	1-10	I	+0.02	2	43	21	5.6	523	2,323
92 Kg.	11-17	II	-0.00	1	84	6	7.7	947	2,212
3/15/57	18-24	III	-0.75	105	92	99	9.5	1,712	2,046
	25-31	IV	-0.2	15	102	16	8.5	744	1,938
	32-34	V	+0.5	1	92	2	7.5	530	2,218

Note: The data recorded for each period represent the average of serial 24-hour determinations.

*Period I = control (mercurial diuretics were given); Period II = prednisone administration; Period III = prednisone and Amphenone administration; Period IV = prednisone administration; Period V = final control (mercurial diuretics were given); Period A = Amphenone administration. See text for dosage.

†a = repeat study; b= no mercurial diuretic given; c = addition of mercurial diuretics every third day; in the other studies mercurials were given during Periods I and V only.

without cirrhosis, revealed a mean ratio for "free" to total ("free" plus "conjugated") of 20 per cent ranging from 1 to 40 per cent (Table III). To check for purity of measured aldosterone, several aliquots of the samples containing aldosterone at the end of the procedure[24] were chromatographed on the "E B" system of Eberlein and Bongiovanni.[27] From this it appeared that most of the samples examined did not contain any of the contaminants found by Nowaczynski and associates[28] (labeled p in Table III). The small amounts of contaminating material found in the few remaining cases (labeled c in Table III) did not interfere with the final quantitative reaction used for aldosterone, as they did not immediately reduce blue tetrazolium.

Prednisone was given before, during, and after Amphenone. All patients received 30 mg. per day, with the exception of Cases 1 and 2 who received 50 mg. per day. Seven patients received Amphenone while 3 others received prednisone alone. The average daily dose of Amphenone was as follows: Case 8, 3.1 Gm.; Case 9, 3.6 Gm.; Case 3, 4.5 Gm.; Case 2, 5 Gm.; Case 1, 5.6 Gm.; Case 10, 5.6 Gm.; and Case 7, 6.0 Gm.

The study was divided into 5 periods: Period I, control (no medication except mercurials), 5 to 10 days; Period II, prednisone, 3 to 12 days; Period III, prednisone and Amphenone, 3 to 7 days; Period IV, final prednisone, 3 to 7 days; and Period V, final control (no medication except mercurials), 2 to 7 days. Mercaptomerin sodium was given once or twice to all patients except Case 9 during Period I and again during Period V. Case 8 left the hospital against advice while receiving prednisone without a final control period. Four patients received a second course of prednisone at a later date. Some of the data on Cases 1, 2, 3, 7, 8, and 10 are included in previous reports from this laboratory.[22, 29]

RESULTS

Metabolic Balance Studies.—Daily results have been averaged for each period and are shown in Table I. The balances are approximate, as stool and insensible losses were not measured. (See Materials and Methods.)

A. Period I, control (no medication except mercurial diuretics): Only slight changes in weight, sodium, potassium, or water balance were found during this period. Weight loss was small and considered insignificant. The greatest weight loss, an average of 0.22 Kg. per day in Case 2, resulted from a 2 Kg. loss in response to mercurial injection. Small weight gains were noted in 3 patients. Mean daily urine sodium was 3.9 mEq. per day, and excluding mercurial responses was 1.9 mEq. per day. Average urine volumes were less than 1,000 ml. per day for 7 of 10 patients, and between 1,000 and 1,500 ml. for the remaining 3.

B. Period II, prednisone. During this period a weight loss greater than 0.25 Kg. per day occurred in 5 of 10 patients (Cases 2, 3, 5, 6, and 9), varying from 1 Kg. in 3 days (Case 2) to 7.1 Kg. in 11 days (Case 9). All patients showed an increase in average urine volume of 350 ml. per day or more (Fig. 1). A decrease of estimated water balance greater than 350 ml. per day occurred in 6 of 8 patients (Cases 3, 5, 6, 8, 9, and 10).

Fluid intake data were incomplete for Cases 1 and 2. A moderate increase of fluid intake occurred only in Cases 4 and 7 (Table I), although only Cases 5 and 6 were restricted in fluid intake. Urine volume bore no relation to urine sodium concentration, except that the 2 patients with volume increases of over 600 ml. per day (Cases 6 and 9) also excreted the greatest amount of sodium.

A brisk sodium diuresis occurred in 3 of 10 patients (Case 5, 33 mEq. per day; Case 6, 61 mEq. per day; Case 9, 68 mEq. per day), and 3 others showed small but definite increases of sodium excretion (Case 1, 5 mEq. per day; Case 3, 7 mEq. per day; Case 7, 14 mEq. per day). The other 4 patients continued to excrete less than one mEq. per day (Fig. 1). Case 2 was the only patient to lose weight without increasing urine sodium. Sodium and water diuresis began from 1 to 4 days after initiation of prednisone, with the maximum effect between the fourth and tenth days. Patient 9 exhibited the most marked diuresis in response to prednisone (Fig. 3). It should be noted that Case 3, the first patient studied, received Amphenone alone on the day prior to the administration of prednisone, and during a later course of prednisone alone he failed to show a natriuresis, so that the effect observed during Period II may have been related to Amphenone administration. An increase in urine sodium can thus be attributed to prednisone in 5 of 10 patients, with a clinically significant loss in 3.

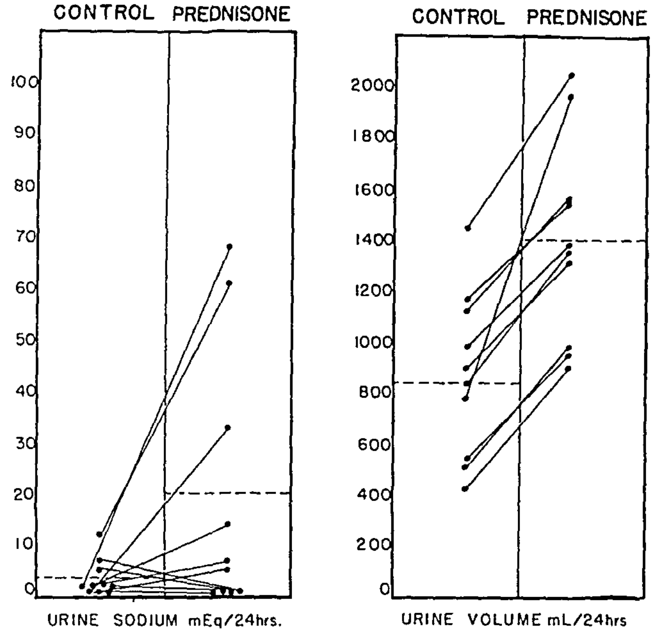

Fig. 1.—Changes in urine volume and urine sodium excretion of 10 patients with cirrhosis and ascites during prednisone administration.

Increases in potassium excretion greater than 25 mEq. per day occurred only in Cases 9 and 10. Correction of potassium excretion using the changes in urine nitrogen* showed that the increased urine potassium in these 2 patients was more than could be accounted for by increased cellular catabolism. Average urine solute concentration rose in some instances and fell in others with no relation to urine volume (Table II).

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*The figures for daily urine potassium excretion were corrected for prednisone-induced cellular catabolism in the following manner. The increase in urine nitrogen (in grams) during the prednisone period multiplied by 2.7 was assumed to be equal to potassium (expressed as milliequivalents) from cellular breakdown.

TABLE II. MEAN URINE AND SERUM SOLUTES FOLLOWING PREDNISONE ADMINISTRATION

PATIENT	PERIOD OF STUDY	URINE VOLUME (ML./24 HR.)	URINE SODIUM (MEQ./24 HR.)	URINE SOLUTES (MOSM./L.)	SERUM SODIUM (MEQ./L.)	SERUM SOLUTES (MOSM./L.)
5	I	516	1	243	127	273
	II	970	33	326	138	284
6	I	1,443	12	255	142	277
	II	2,069	61	239	140	290
9	I	783	2	--	135	288
	II	1,966	68	--	139	296
7	I	1,115	2	245	135	284
	II	1,566	14	252	136	291
8	I	988	7	-	132	274
	II	1,384	1	-	138	288
10	I	523	2	492	131	285
	II	947	1	480	134	294

Note: The urine data recorded for each period represent an average of serial 24 hour determinations while the serum data represent the last determination during the period.

C. Period III, Amphenone and prednisone. During Amphenone administration 4 of 7 patients showed weight loss of from 0.17 to 0.75 Kg. per day and an increase in urine sodium excretion of from 18 to 104 mEq. per day. Case 2 continued to excrete less than 1 mEq. of sodium per day while losing weight, and Case 1 increased sodium excretion slightly (to 13 mEq. per day) while gaining weight. Although a marked natriuresis occurred in Case 9 during prednisone administration, the urine sodium excretion reached its maximum on the first day of Amphenone administration and then fell off in spite of continued therapy. By the end of the Amphenone period, he was clinically free of ascites (Fig. 3). Fluid balance became significantly more negative only in Case 10, who was the only patient to receive Amphenone in full 6 Gm. doses for more than 3 days. Average urine potassium excretion increased from 1 to 25 mEq. per day in all patients but Case 1.

Period II was continued for at least 8 days for the majority of patients, permitting evaluation of the effect of prednisone administration. However, the possibility that the 3 day course of prednisone used in Cases 1, 2, and 3 could have initiated a diuresis made evaluation of a subsequent Amphenone effect difficult, as a diuresis began on the third or fourth day of prednisone administration in some patients. To define the extent of the prednisone effect, these 3 patients were each given a second course of prednisone alone 3 weeks to 6 months later. After 5 to 8 days of prednisone, Case 3 (in 2 separate trials) showed no response in sodium or fluid balance, while Case 2 (in one trial) again showed an increase in urine volume and weight loss without an increase in sodium excretion. Case 1 showed a decrease in ascites and weight loss, however, urine collections were incomplete.

Thus an unequivocal natriuresis in response to Amphenone occurred in 4 of 7 patients (Cases 3, 7, 8, and 10). Interpretation of the response in 2

TABLE III. URINE ALDOSTERONE EXCRETION

PATIENT	PERIOD	DAY	URINE ALDOSTERONE* (μG/24 HR.)			PER CENT FREE	P
			FREE	CONJUGATED	TOTAL		
A. Patients With Cirrhosis							
9	I	Mean	5^{c}	10	15	33	
	II	15	9	10	19		
		16	4.5	12^{p}	16		
		17	0	7.5	7.5		
		Mean	4.5	9.8	14	32	
	III	18	—	—	6		
		19	—	—	7		
		20	—	—	4.5		
		Mean	—	—	5.8	39	
	III	23	12^{p+}	20^{p+}	32^{+}	37	
	IV	24	28	25^{p}	53		
		25	10	28^{p}	38		
		26	15	22^{p}	37		
		Mean	18	25	43	42	
	V	32	5	40^{p}	45		
		34	30	16^{p}	46		
		Mean	17	28	45	38	
10	I	Mean 1 to 10	18^{c}	25^{p}	43	42	
	II	Mean 11 to 17	15^{c}	28^{p}	43	35	
	III	Mean 18 to 24	2	2	4	50	
	IV	Mean 25 to 31	0	4	4	—	
	V	Mean 32 to 34	26^{p}	23^{p}	49	53	
1	I	2	35	20	55		
		3	5.5	2.5	8		
		5	17	2	19		
		Mean	19	8	27	70	
	II	7	5	26	31		
		8	8	0	8		
		9	35	4.5	39		
		Mean	16	9.8	26	61	
	III	10	0.5	2.5	3		
		11	2.0	0	2		
		12	2.5	1.5	4		
		Mean	1.6	1.3	3	53	
	IV	13	6.5	—	—		
		14	4.0	—	—		
		Mean	5.2	—	—		
2	I	1	36	14	50		
		3	25	22	47		
		6	21	18	39		
		Mean	27	18	45	60	
	II	8	59	10	69		
		9	30	—	—		
		10	15	2	17		
		Mean	35	6	43	81	
	III	11	2.5	4	6.5		
		12	4.0	5	9.0		
		13	0.5	5	5.5		
		Mean	2.3	4.6	7.0	33	
	IV	14	5	25	30		
		15	20	20	40		

TABLE III—CONT'D

DIAGNOSIS AND NUMBER OF PATIENTS	URINE ALDOSTERONE (µG/24 HR.)			PER CENT FREE
	FREE	CONJUGATED	TOTAL	
B. Patients Without Liver Disease				
Edema of unknown origin (1)	0.5	65	65.5	1
Congestive cardiac failure (1)	2	6	8	25
Pregnancy (1)	0.5	6	6.5	8
Unclassified edema (1)	1	3	4	25
Normal men after 1 week of sodium restriction (pool)	5†	7†	12†	41
Patients with Addison's disease given intravenous *aldosterone (pool)*	2†	8†	10†	20

Note: all figures represent determinations performed on 24 hour urine samples except for Subject 10 (Periods I to V), Subject 9 (Period I), and 2 pools of patients without liver disease where the determinations were performed on pooled daily aliquots.

*Key to symbols: c = sample found to be contaminated after additional chromatography on E.B. paper system; p = sample found to be pure after additional chromatography on E.B. paper system; + = figures not used in calculating mean values since Amphenone dosage was reduced to 1.5 Gm. per day during Days 21 to 23.

†Data expressed as micrograms of aldosterone per liter.

(Cases 1 and 9) was complicated by a concomitant prednisone effect, and no natriuresis occurred in another (Case 2). Only one patient (Case 10) developed a clinically significant loss of ascites during Amphenone administration.

D. Period IV, final prednisone. During this period, daily urine volume and electrolyte excretion tended to return to Period I values; however, patients who exhibited a marked sodium diuresis in Period II continued to lose sodium and water until ascites was no longer evident.

Frequently, the most striking natriuresis due to Amphenone occurred 4 to 5 days after the first dose, which was after the drug had been discontinued in most cases. In Case 10 the maximum response was observed on the fifth day while he was still receiving 6 Gm. of Amphenone daily (Fig. 2), indicating that the delayed natriuresis observed in the other patients was probably not due to abrupt discontinuance of Amphenone.

E. Period V, final control. Daily balance studies approximated those of Period I except that mercaptomerin induced a greater increase in water and electrolyte excretion than during Period I.

Aldosterone Excretion.—Aldosterone excretion was above normal during the control period in all 4 patients studied; however, most of these values did not exceed those found in normal subjects on a sodium restricted diet (Table III).[30]

Prednisone caused no consistent change in total aldosterone excretion nor in the ratio of "free" to "conjugated" fractions. In Case 2 there was a fall in aldosterone excretion over a 3 day period while on prednisone (Fig. 5); however, this was not accompanied by an increase in urinary sodium. The

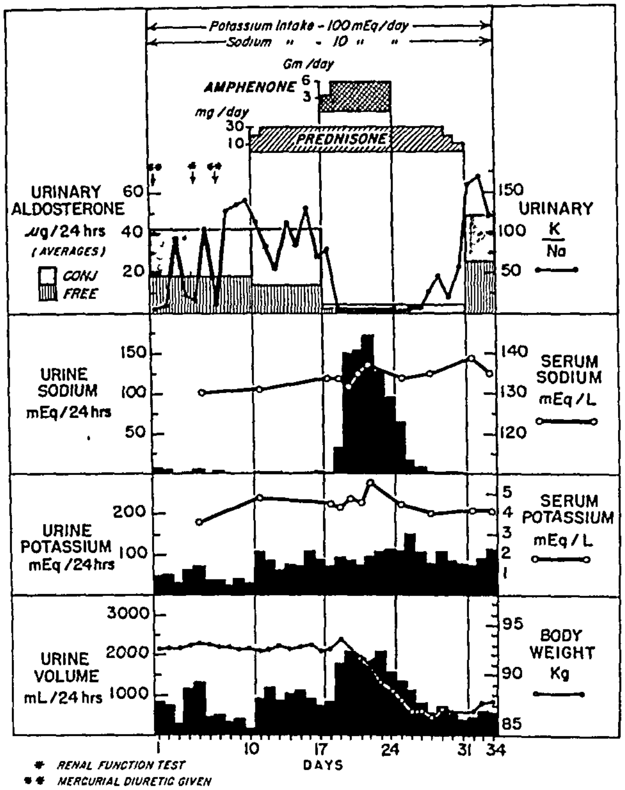

Fig. 2

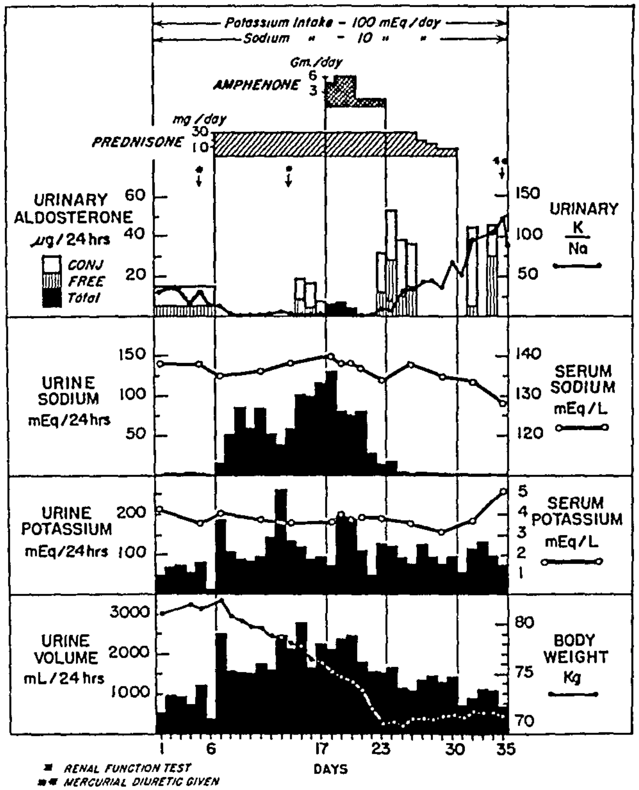

Fig 3.

Fig. 2.—Balance study of a patient who showed a marked sodium diuresis in response to Amphenone administration (W W, a 48-year-old man with portal cirrhosis and ascites)

Fig. 3.—Balance study of a patient who showed a marked sodium diuresis in response to prednisone alone, with no enhancement associated with the addition of Amphenone He was clinically free of ascites by the end of the Amphenone period. (M K, a 40-year-old man with portal cirrhosis and ascites)

massive sodium diuresis exhibited by Case 9 in response to prednisone administration was not accompanied by a change in aldosterone excretion (Fig 3) Thus, the natriuresis induced by prednisone in this case could not be related to a fall in aldosterone excretion

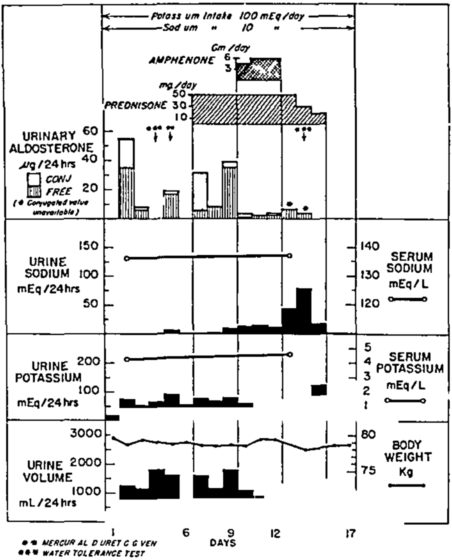

Fig 1—Balance study in a patient who showed a slight sodium diuresis in response to prednisone and Amphenone (J A a 62 year old man with portal cirrhosis and ascites)

Amphenone administration caused a 60 to 90 per cent decrease in aldosterone excretion in all 4 patients in whom it was measured (Table III), however, this was not always associated with an increase in sodium excretion (Figs 2 to 5) Case 2 exhibited no increase in sodium excretion despite a decrease in aldosterone excretion, and in Case 9 the decrease in aldosterone excretion following Amphenone actually was associated with a relative fall in a previously elevated sodium excretion (Fig 3) An increased sodium excretion was associated with the fall in aldosterone excretion in Cases 1 and 10 However, in subsequent studies, Case 1 exhibited a diuretic response to prednisone alone Thus, of the 4 patients so studied, Case 10 was the only one in whom the natriuresis could be definitely attributed to a decreased aldosterone effect

The aldosterone excretion of patients without liver disease on an unrestricted diet is 0 to 15 µg per 24 hours with a mean of 5, using the method

TABLE IV. CHANGES IN SERUM CONSTITUENTS FOLLOWING PREDNISONE AND AMPHENONE ADMINISTRATION

PATIENT	SERUM SODIUM (mEq./L.)			SERUM POTASSIUM (mEq./L.)			SERUM CHLORIDE (mEq./L.)			BLOOD HEMATOCRIT (%)			SERUM ALBUMIN (GM. %)			SERUM BILIRUBIN (MG. %)		
	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
1 (1)	136	—	137	4.3	—	4.6	94	—	99	37	—	—	4.0	—	3.6	1.6	—	0.1
(2)	124	136	—	5.5	5.3	—	93	90	—	37	38	—	2.8	2.8	—	2.3	1.9	—
3 (1)	136	—	131	4.6	—	5.8	97	—	103	25	—	32	3.7	—	3.7	1.2	—	0.8
(2)	138	139	—	4.0	4.0	—	105	99	—	26	30	—	3.8	4.1	—	—	—	—
2	138	138	139	4.0	4.3	4.5	92	98	101	—	—	—	4.6	—	4.7	0.8	—	0.4
4 (1)	136	132	—	4.0	5.7	—	96	99	—	31	31	—	4.2	—	—	5.1	3.4	—
(2)	129	136	—	3.6	4.4	—	97	100	—	27	30	—	3.1	2.8	—	3.4	3.7	—
7	135	136	132	4.2	5.4	5.4	90	97	92	31	29	28	2.1	2.5	2.6	0.2	0.2	0.1
8	132	138	137	3.4	3.9	3.8	99	119	103	32	33	35	3.2	2.9	2.8	2.0	1.3	1.2
5	127	138	—	4.9	3.8	—	95	105	—	33	35	—	2.8	3.6	—	2.6	1.3	—
6	142	140	—	3.8	3.8	—	95	106	—	34	36	—	2.8	3.0	—	2.3	1.5	—
9	135	139	137	4.1	3.6	3.8	106	102	104	36	36	35	1.9	3.1	—	4.3	3.3	3.5
10	131	134	134	4.8	4.5	4.5	102	102	109	30	34	33	3.6	—	3.9	1.8	1.5	1.5

Note: (a) 1 (2), 3 (2), and 4 (2) refer to a repeat study in the same patient. (b) I, II, and III refer to periods of study as described in the text.

described above (Table III). With sodium restriction, the aldosterone excretion rises to a level of 10 to 45 μg per 24 hours by the third to fourth day and subsequently may fall to a level slightly above initial values.[3] Marked individual variation is usually observed.

The mean "free" urine aldosterone ranged from 32 to 81 per cent of the total ("free" plus "conjugated"), which may be contrasted with a mean of 20 per cent for patients without liver disease (Table III). This value for normal subjects is in agreement with the results of Axelrod and associates[8] using a bio-assay technique. The ratio remained relatively constant in each patient throughout the study, although there was a trend toward normal in Case 2 during Periods III and IV.

Urine K to Na ratios,* calculated for Cases 9 and 10 (Figs. 2 and 3), could not be closely correlated with aldosterone excretion. This lack of correlation had been observed in normal subjects on unrestricted[24] and on sodium-restricted diets,[30] using the same method for aldosterone determination. This is in contrast with the experience of Luetscher and Curtis, using the bio-assay technique.[31]

Serum Electrolytes and Related Measurements.—The effect of prednisone and Amphenone on serum electrolytes and related measurements is seen in Table IV. During prednisone administration (Period II) the serum sodium concentration rose 7 to 12 mEq. per liter in 3 instances, 3 to 7 mEq. per liter in 3, and remained essentially unchanged in 4 (the latter in patients with relatively normal serum sodium concentrations). A fall of 4 mEq. per liter was noted in one instance, Case 4 (2). The 3 serum sodium concentrations between 124 and 130 mEq. per liter at the end of the control (Period I) were above 135 mEq. per liter after prednisone (Period II) (Fig. 6). A rise of serum sodium concentration began in Case 5 within 2 days, before changes in sodium or fluid balance were evident. With this data it is impossible to determine whether these changes in serum sodium concentration were related to an internal shift of fluid and electrolytes or to increased urine volume and increased insensible fluid loss. Serum solute concentration rose in all 6 patients so measured (Table II).

A corresponding but less significant rise occurred in total serum protein and hematocrit in response to prednisone administration. There were no consistent changes in nonprotein nitrogen or carbon dioxide content. Serum chloride concentration tended to rise during prednisone administration in all but 3 instances. Serum potassium concentration was normal in most instances and there were no consistent deviations. Serum bilirubin concentration tended to fall slightly while serum albumin concentration tended to rise during prednisone administration. There was no correlation between pretreatment serum albumin or bilirubin concentrations and a response to prednisone, and improvement in these values was maintained through the final control period.

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*See footnote on page 400.

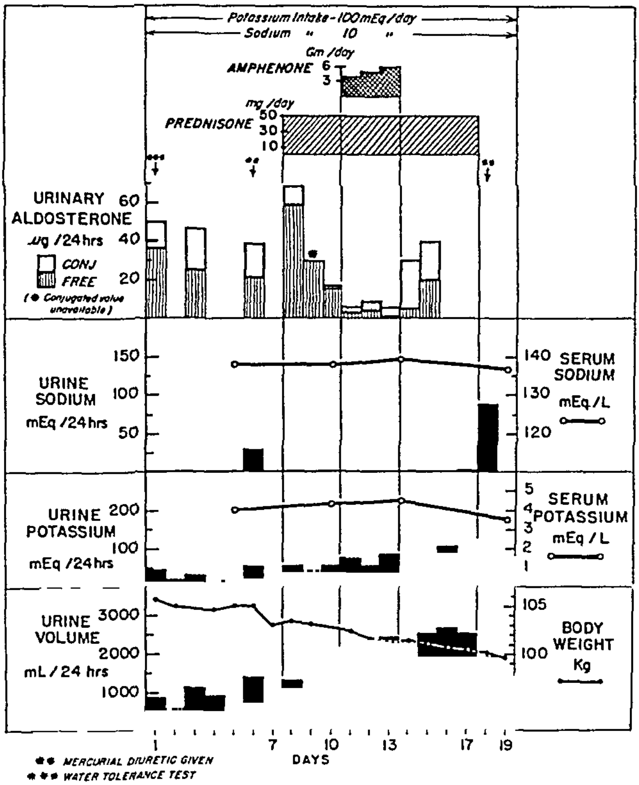

Fig. 5.—Balance study in a patient who showed no sodium diuresis in response to prednisone or Amphenone administration. (J. O., a 40-year-old man with portal cirrhosis and ascites.)

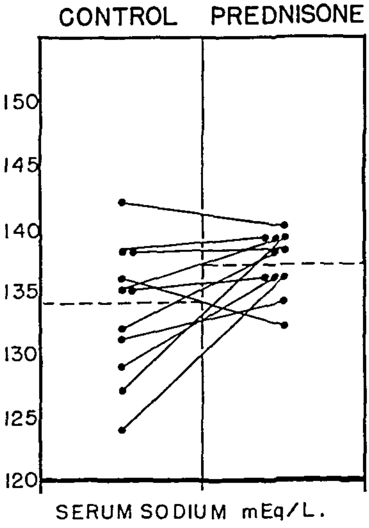

Fig. 6.

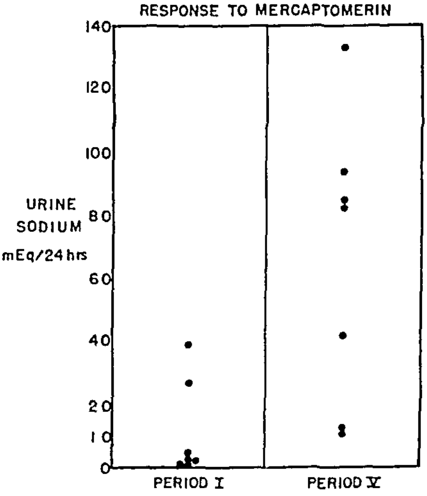

Fig. 7.

Fig. 6.—Change in serum sodium in response to prednisone administration. Eleven studies in 10 patients. (See Table IV.)

Fig. 7.—Urine sodium excretion in response to 2 ml. mercaptomerin subcutaneously before and after prednisone administration.

The administration of Amphenone for short periods produced no striking or consistent changes, however, 3 patients showed a slight rise in serum potassium and a fall in serum sodium No significant alteration in serum albumin or bilirubin concentration or cephalin flocculation was noted

Response to Mercurial Injections—The majority of patients exhibited a poor response to mercaptomerin during the initial control period although 3 (Cases 5, 6, and 8) excreted over 20 mEq of sodium on the day of injection A small increase in sodium excretion was seen on the day after injection in several instances

Following discontinuance of prednisone (Period V), mercaptomerin administration increased urine sodium excretion from 2 to 100 times the Period I values (Fig 7) This increased sensitivity usually decreased rapidly with repeated mercurial injections In 3 patients (Cases 3, 4, and 7) who did not achieve a significant sodium diuresis on prednisone, the addition of mercurials in 2 or 3 day intervals for a period of 9 to 12 days, although transiently increasing urinary sodium, did not cause a significant weight loss or loss of ascites

Hypochloremia and decreased glomerular filtration rate both may be factors in mercurial unresponsiveness[32, 33] Steroids are known to increase the glomerular filtration rate, although this may be inconstant in patients with cirrhosis[34] The serum chloride was higher during the final control period in each patient except Case 7, however, there was no consistent correlation between the amount of change in serum chloride levels and the degree of responsiveness to mercaptomerin

Clinical Observations and Complications—Coincident with prednisone administration, a feeling of well being and euphoria was experienced by all patients, however, there were no serious psychologic complications Appetite increased markedly in some patients Complaints of between meal hunger were treated with interval feedings subtracted from the daily diets and with aluminum hydroxide gel* Antrenyl,†[35] an anticholinergic drug, was added in some instances No evidence of peptic ulceration or of upper gastrointestinal hemorrhage was observed, although bleeding in small amounts from rectal hemorrhoids occurred in 3 patients An increase in peripheral venous distention was noted sporadically, and a softening of the abdomen was noted occasionally in patients with tense ascites, prior to fluid balance changes

Serious complications ending fatally developed in 2 instances Both occurred following repeated courses of prednisone in severely debilitated patients who had clinical and laboratory evidence of far advanced liver disease with recent episodes of impending hepatic coma Both patients were severely ill with grave prognoses at the time of the repeated courses of prednisone, and it is felt that steroids masked the systemic response to infection making diagnosis difficult and may have potentiated the virulence of the infections by impairment of host resistance

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*Maalox William H Rorer Inc Philadelphia Pa

†We are indebted to Ciba Pharmaceuticals Inc Summit N J for supplies of Antrenyl

Amphenone produced a syndrome clinically indistinguishable from impending hepatic coma in the 2 patients in whom it was given alone (Cases 3 and 10). Toxicity within 1 to 4 hours after the initial dose forced discontinuance of the drug in each case. These symptoms were less severe and their onset was delayed until the second or third day when prednisone was given prior to and during Amphenone administration. Even on the combination of drugs, 6 of 7 patients developed symptoms of impending hepatic coma, while Case 10 developed no toxic symptoms after 6 days of prednisone (30 mg. per day), and Amphenone (6 Gm. per day). This reaction to Amphenone and its relation to hepatic function have been discussed in previous reports.[17, 22, 29] There was no evidence of aggravation of pre-existing liver disease as a result of prednisone or Amphenone administration.

DISCUSSION

The findings reported in this study supplement previous work showing that Amphenone inhibits the adrenal cortical biosynthesis of aldosterone[14, 16, 17, 19] and other steroids[14, 15, 17, 18] in man. Increased sodium excretion accompanied Amphenone administration in 5 of 7 patients to whom it was administered; however, sodium loss could be correlated definitely with an Amphenone effect in only 4. The fall in urine aldosterone (observed in all 4 patients in whom it was measured) during Amphenone administration was correlated with increased urine sodium in 2 instances, but this natriuresis could be attributed definitely to aldosterone inhibition in only one case. Toxicity prevented prolonged Amphenone administration, thus limiting prolonged study and clinical usefulness.

The elevated aldosterone excretion observed during the control period is in agreement with previous work with cirrhotic patients[6-8] and is consistent with the findings of increased aldosterone excretion in other edematous states.[6, 36, 37] Nevertheless, the demonstration that reduction of dietary sodium in normal subjects will cause an increased aldosterone excretion, followed several days later by a decrease,[30, 38, 39] makes interpretation of our results difficult in view of the prolonged strict dietary sodium restriction. Thus, although aldosterone excretion during the control period was elevated in all patients measured, it was often not above that seen in patients without liver disease on a sodium-restricted diet (Table III).[30]

A sodium diuresis occurred in response to prednisone alone in 5 of 10 patients, and was clinically significant in terms of loss of ascites in 3. Aldosterone excretion (measured in 4 patients with and without increased urine sodium) could not be correlated with the prednisone-induced natriuresis; in fact, a marked diuresis occurred over a 10-day period in one patient, while the aldosterone excretion remained unchanged. This finding is not in agreement with the work of Vesin and co-workers[40] who observed a marked fall in aldosterone excretion in conjunction with a prednisone diuresis in one patient, nor with that of Muller and associates[11] who observed a slight decrease in aldosterone excretion in a patient with cirrhosis receiving prednisone, although he did attribute the diuresis to other causes as well as to aldosterone reduction However, our findings are consistent with the observation that a prednisone induced diuresis in a patient with nephrotic syndrome was not associated with a fall in urine aldosterone[4]

The mechanisms controlling aldosterone secretion in man under physiologic conditions are poorly understood Production of aldosterone appears to be controlled partly by changes in extracellular fluid volume,[43, 44] partly by blood potassium concentration,[31, 45, 4] and partly by adrenocorticotrophic hormone (ACTH),[47, 49] although it is evident that not all changes in aldosterone excretion can be accounted for by these known mechanisms[50, 1] No clear relationship is known between any one of these controlling factors and the increased aldosterone excretion observed in patients with cirrhosis and ascites Thus, there is no increase in serum potassium[3] Moreover, measurable extracellular fluid volume is not decreased, even on a sodium restricted diet,[52] and expansion of the extracellular fluid by infusions does not consistently cause diuresis[53] Aldosterone does not seem to be ordinarily regulated by ACTH,[4] and inhibition of ACTH with cortisone or prednisone has not produced a constant diuresis[31, 51, 55] In addition, our results fail to show a consistent fall in aldosterone excretion in response to prednisone administration The demonstration that urine aldosterone excretion increases following abdominal paracentesis in cirrhosis[7, 5] is in accord with the concept of aldosterone secretion regulated by fluid volume changes, in view of the observation that paracentesis is a "dehydrating" procedure in cirrhosis (i e, a decrease in plasma and nonascitic extracellular fluid volumes)[11, 9] Thus, aldosterone secretion in cirrhosis may increase in response to a diminished "effective but unmeasurable" extracellular fluid volume or to diminished intravascular fluid volume which is secondary to the transudation of fluid into the abdominal cavity The lack of marked increases in aldosterone excretion noted in our patients during control periods may reflect the small amount of ascites formation Although our data suggest a relationship between aldosterone excretion and sodium retention in these patients, the absence of complete correlation would indicate that other factors also may be involved Refinement in technique may be needed to elucidate the mechanism of increased aldosterone excretion in cirrhosis with ascites

The present observations raise the question of whether the amounts of aldosterone measured in the urine actually reflect quantitatively the rate of secretion of the hormone by the adrenal cortex As the amount of aldosterone excreted in the urine represents only a small fraction of adrenal production the significance of observations based on urinary excretion is somewhat limited Work with animals has shown that large increases in aldosterone as measured in the adrenal vein may not be reflected in the peripheral blood, and the amount appearing in the urine has been estimated to be as small as 1 per cent of adrenal secretion[58] In man, using labeled aldosterone, the proportion of infused aldosterone excreted in urine was 4 per cent[59] A previous discussion has emphasized the reliability of the analytic procedure used in the present study[24]; however, it is possible that in patients with cirrhosis, the impaired liver function might allow a greater than normal proportion of adrenal aldosterone production to be excreted unaltered in the urine. Suggestive evidence that cirrhosis alters aldosterone metabolism arises from the increased proportion of urine aldosterone present in the free or readily hydrolyzable form. This is in accord with the results of Axelrod and associates[8] and would indicate that the metabolism of aldosterone and of cortisol do not necessarily follow similar pathways. Indeed, Englert and associates[60] showed that, although the reduction of cortisol was impaired in liver disease, the conjugation of its metabolites proceeded unimpaired. A more definite answer to these problems must await a satisfactory method of measuring plasma aldosterone.

In contrast to the variable effect of prednisone on aldosterone and sodium excretion, a uniform effect on urine volume was observed. All patients developed increased urine volume during prednisone administration, while only 5 of 10 developed increased urine sodium excretion, and this natriuresis was not directly related to the degree of increase in urine volume. These findings are consistent with the results of others.[34, 54, 55]

Continued administration of adrenal steroids to normal men influences the renal tubular reabsorption of water causing an increase in the maximal rate of water diuresis, which is unrelated to changes in glomerular filtration rate, solute excretion, or antidiuretic hormone effect.[61] A similar mechanism may be responsible for the diuretic effect of prednisone in cirrhosis with ascites. Endogenous creatinine clearance during prednisone administration[34] suggests that glomerular filtration rate alterations are not related to the diuresis of these patients. The increased urine volume did not seem to represent purely a solute diuresis, for the urine solute concentration in some patients receiving prednisone decreased, indicating that the rise in solute output was less than the rise in urine volume. The rise in serum solute concentration following prednisone administration is consistent with a decreased antidiuretic hormone effect; however, the inadequacy of this postulate is indicated by the finding of some daily urine solute concentrations of 400 to 500 mOsm. per liter and over.* In fact, the rise in serum solute concentration would be expected to stimulate secretion of antidiuretic hormone. Thus, the concept of an altered renal tubular reabsorption of water unrelated to changes in glomerular filtration, solute excretion, or antidiuretic hormone effect could also apply to the patients in this study. In acute studies on normal subjects, sodium diuresis invariably accompanied water diuresis due to adrenal steroid administration,[61] while only half of the patients with cirrhosis exhibited an increased sodium excretion. This is in accord with previous observations that patients with cirrhosis and ascites may retain sodium avidly even in the face of strong stimuli for sodium excretion. The data presented in this study are insufficient to allow a final conclusion concerning the method by which prednisone affects water and sodium excretion in patients with cirrhosis and ascites, however, the data do not support the concept[40] that a decreased aldosterone excretion alone is involved in the natriuresis caused by prednisone

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*Antidiuretic hormone is necessary for a concentrated urine under ordinary circumstances; however, Berliner[62] has shown that a concentrated urine may be formed in the absence of antidiuretic hormone under certain conditions when there is a decreased volume of urine reaching the concentrating segments of the renal tubules.

Amphenone administered in small doses to patients with liver disease causes a syndrome clinically similar to impending hepatic coma, and the coadministration of prednisone was necessary to decrease the severity of this reaction The serious complications noted in 2 patients following repeated courses of prednisone warrant the use of caution when administering this drug to severely debilitated patients It should be emphasized that the patients in this study were selected for their lack of response to conventional diuretic measures, and many were seriously ill At present, the adverse manifestations of prednisone and Amphenone are a serious limitation to their clinical effectiveness for this group of patients

SUMMARY

1 Partial inhibition of adrenal steroid production by Amphenone in patients with cirrhosis and ascites who were maintained on prednisone and sodium restriction, was associated with increased urine sodium excretion in 5 of 7 cases, although a clinically significant loss of ascites occurred in only one A complete inverse correlation between sodium and aldosterone excretion was not observed, and it is suggested that other factors as well as aldosterone secretion are operative in causing the sodium retention of these patients In addition, the data indicate that patients with cirrhosis may have an impaired ability to excrete conjugated aldosterone

2 Prednisone was found consistently to cause a water diuresis when administered to patients with cirrhosis, and this was associated with an increased response to mercurial diuretics and frequently a rise in serum sodium Increased sodium excretion followed prednisone administration in 5 of 10 cases but was clinically significant in only 3 and could not be correlated with decreased aldosterone excretion

3 The adverse neurologic manifestations following Amphenone administration to patients with cirrhosis even though ameliorated by concomitant prednisone administration, create a severe limitation to its clinical usefulness Prolonged prednisone administration to these patients with cirrhosis was associated with overwhelming infection in 2 instances

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ORNITHINE CARBAMYL TRANSFERASE IN DOG SERUM ON INTRAVENOUS INJECTION OF ENZYME, CHOLEDOCHUS LIGATION, AND CARBON TETRACHLORIDE POISONING

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IN THE mammal, ornithine carbamyl transferase (OCT)[1] has hitherto been found only in liver cells[2] It occurs normally in minute amounts in blood serum and is often increased in liver disease This rise may be of diagnostic and prognostic significance[3] The aim of the present investigation was to elucidate some factors with an important bearing on the diagnostic evaluation of serum OCT

MATERIAL AND METHODS

The experimental animals were all healthy mongrel dogs, weighing between 13 5 and 21 kilograms, that had been dewormed and vaccinated against distemper

Operations—All dogs were anesthetized with Nembutal 0 035 Gm per kilogram of body weight intraperitoneally, and received 600 000 I U procaine penicillin by intramuscular injection to prevent infection Intubation was done in order to obviate anoxia The operations were performed by Dr G Jonson and Dr D Hallberg

Ligation of the Common Bile Duct—After opening the abdomen by a midline incision, the choledochus was identified, ligated, and then cut 3 to 5 cm from the duodenal papilla The belly was closed within 30 to 60 minutes All dogs recovered consciousness within 6 hours after the onset of anesthesia, and were on their feet the day after operation

At subsequent necropsy the bile duct obstruction was checked and found to be complete

Control animals were subjected to the same operative procedure with the exception of ligation and division of the choledochus

Reoperation—The dogs that had undergone choledochus ligation were submitted, 15 to 17 days later, to a second operation at which time the continuity of the common bile duct was restored by means of a Teflon tube This operation lasted from 2 to 3 hours and greatly impaired the general condition of the jaundiced animals In 2 of them fluid had to be administered intravenously

Carbon Tetrachloride Poisoning—In 4 anesthetized dogs, 1 ml of carbon tetrachloride per kilogram of body weight was instilled through a gastric tube These animals recovered consciousness within 4 hours after the onset of anesthesia Their condition had deteriorated and they were unwilling to get on their feet

Preparation of Liver Extract Containing OCT—The abdomen of anesthetized dogs was opened rapidly and 100 Gm of liver tissue removed, then cooled with ice to 0° C All further manipulations were done at that temperature The liver tissue was homogenized, together with 300 ml distilled water, in a Waring blendor This treatment releases OCT from the mitochondria The homogenate was centrifuged at 20 000 g for 30 minutes The light red slightly opalescent fluid was used within 24 hours The extract had a high OCT activity (Table III)

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Tissue Homogenates.—Tissue homogenates of different organs were prepared from healthy dogs that had been killed by intravenous injection of air during anesthesia. All specimens were taken within 5 minutes after death and treated as above but not centrifuged.

OCT Activity[*].—In serum or in tissue homogenates OCT activity was determined by incubation with citrulline-carbamyl-C^{14} in arsenate buffer.[4] The amount of $C^{14}O_2$ formed is proportional to the OCT activity. Results were expressed in micromoles of $C^{14}O_2$ per 0.5 ml. of serum or 1 Gm. of tissue.

Other Determinations.—Glutamic oxalacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) were assayed by the method of Reitman and Frankel.[5] Results were expressed in Karmen units.[6]

RESULTS

OCT Content.—The OCT content of different organ homogenates is detailed in Table I. No OCT activity exceeding that found in normal serum (0.024 ± 0.010 μM of $C^{14}O_2$ per 0.5 ml. of serum) was demonstrable in brain, lung, spleen, pancreas, kidney, striated muscle, or heart muscle. Gall bladder bile, stomach, cecum, and ileum showed enzyme activities equivalent, respectively, to 0.06, 0.74, 2.60, and 43.0 μM of $C^{14}O_2$ per 1 Gm. of tissue or per 1 ml. of bile. The corresponding value for liver was 2,900 μM of $C^{14}O_2$.

TABLE I. OCT CONTENT IN THE DIFFERENT ORGANS FROM DOG. ALL VALUES ARE THE MEANS OF DETERMINATIONS FROM 2 ANIMALS

ORGAN	OCT ACTIVITY IN μM $C^{14}O_2$ PER Gm. TISSUE
Brain Lung Spleen Pancreas Kidney Striated muscle Heart muscle	<0.01
Blood serum	0.04
Gall bladder bile	0.06
Stomach (pyloric portion)	0.74
Duodenum	36.0
Ileum	43.0
Cecum	2.6
Liver	2,900.0

To rule out intestinal contents as the source of OCT activity, both washed and unwashed specimens from stomach, ileum, and cecum were incubated, but no verifiable difference in enzyme activity emerged.

OCT Levels.—The 24-hour OCT levels in dog serum are set forth in Table II. In addition to OCT, both GOT and GPT were assayed since they were consistently used as reference enzymes. Blood samples were taken here, as in all experiments, by puncture of a leg vein. No 24-hour variation of significance was observed.

Elimination Rate of Liver Extract.—The elimination rate of intravenously administered liver extract containing OCT is shown in Table III. The mean for the 3 dogs will be found in Fig. 1. The 3 different liver extracts had about the same OCT content (660 to 730 µM of $C^{14}O_2$ per 1 ml.). Their GOT and GPT contents, which were not determined, probably varied substantially.

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[*] In an earlier paper[1] the concentration of citrulline-carbamyl-C^{14} was erroneously given as 1 μM per milliliter. This should be 10 μM per milliliter of final incubation mixture.

TABLE II. The 24 Hour Serum Levels of OCT, GOT, and GPT in 2 Dogs. Normal Values: OCT, 0.024 ± 0.010 µM of $C^{14}O_2$ per 0.5 ml. Serum, GOT, 18.5 ± 10.4 Units, GPT, 23.3 ± 10.0 Units. All Normal Values are From 15 Dogs

DOG NO.	DAY	TIME	OCT IN µM $C^{14}O$ PER 0.5 ML. SERUM	GOT (KARMEN UNITS)	GPT (KARMEN UNITS)
1	1	9:00 A.M.	0.011	20	16
		1:00 P.M.	0.018	30	25
		5:00 P.M.	0.018	25	16
		9:00 P.M.	0.010	25	15
	2	2:00 A.M.	0.018	25	16
		9:00 A.M.	0.018	30	20
2	1	9:00 A.M.	0.039	28	15
		1:00 P.M.	0.030	25	20
		5:00 P.M.	0.029	25	20
		9:00 P.M.	0.040	23	22
	2	2:00 A.M.	0.040	25	22
		9:00 A.M.	0.044	25	20

TABLE III. Serum Levels of OCT, GOT, and GPT After Intravenous Injection of Liver Extract. The Liver Extracts Administered in Dogs 3, 4, and 5 Had an OCT Activity Equivalent to, Respectively, 680, 660, and 730 µM of $C^{14}O$ per Milliliter. All Animals Were Injected With 2 ml. Extract per Kilogram Body Weight on Day 1 at 8:00 A.M. Immediately After the First Sample Had Been Taken

DOG NO.	DAY	TIME	OCT IN µM $C^{14}O$ PER 0.5 ML. SERUM	GOT (KARMEN UNITS)	GPT (KARMEN UNITS)
3	1	8:00 A.M.	0.018	25	40
		10:00 A.M.	7.60	700	400
	2	10:00 A.M.	0.88	40	130
	3	10:00 A.M.	0.119	22	78
	4	10:00 A.M.	0.070	28	72
	6	10:00 A.M.	0.011	12	29
4	1	8:00 A.M.	0.038	26	32
		10:00 A.M.	8.30	1,300	1,600
	2	10:00 A.M.	0.79	90	1,000
	3	10:00 A.M.	0.370	18	715
	4	10:00 A.M.	0.044	12	440
	6	10:00 A.M.	0.037	25	160
5	1	8:00 A.M.	0.036	51	38
		10:00 A.M.	11.7	700	1,070
	2	10:00 A.M.	2.60	85	630
	3	10:00 A.M.	0.420	28	450
	4	10:00 A.M.	0.162	60	240
	6	10:00 A.M.	0.035	90	190

OCT was so eliminated from the blood stream that its serum level, measured 1, 2, and 3 days after injection of the liver extract, amounted to 14, 3, and 1 per cent, respectively, of the value determined 2 hours after the injection. The corresponding values for GPT were 59, 42, and 23 per cent, respectively. Serum GOT had fallen to 8 per cent after one day and was normal on the second day.

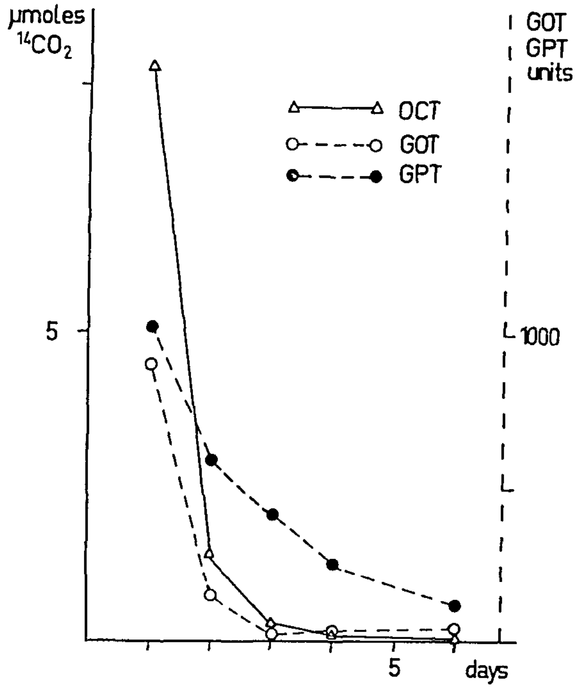

Fig. 1.—Elimination of intravenously administered liver extract on Day 1 (2 ml. per kilogram body weight). Means in 3 dogs from Table III. GOT and GPT in Karmen units.

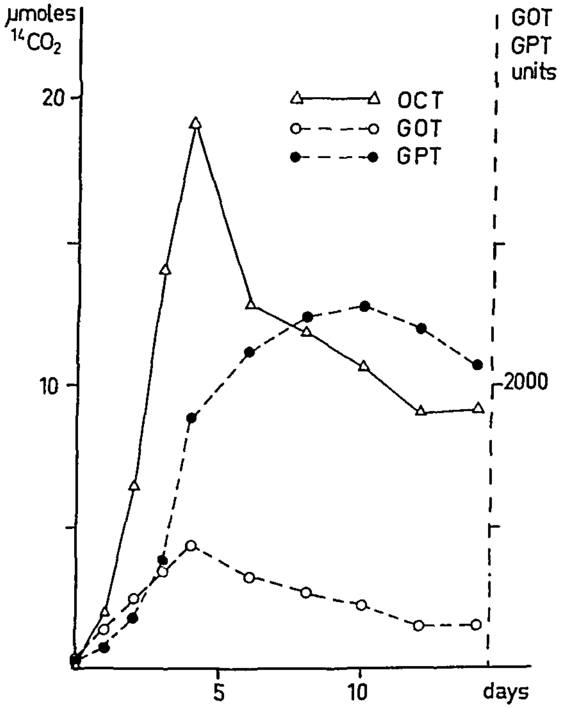

Fig. 2.—Serum OCT, GOT, and GPT after ligation of common bile duct (Day 0). Means in 3 dogs from Table IV. GOT and GPT in Karmen units.

OCT Content of Serum Following Choledochus Ligation.—The OCT of serum following choledochus ligation and in the controls is given in Table IV, the mean for the ligated dogs in Fig. 2.

TABLE IV. SERUM LEVELS OF OCT, GOT, AND GPT AFTER LIGATION OF CHOLEDOCHUS (DOGS 6, 8, AND 10) AND IN CONTROL (DOGS 7, 9, AND 11). THE OPERATIONS WERE PERFORMED ON DAY 0 BETWEEN 1.00 AND 5.00 P.M. SAMPLES WERE TAKEN AT 10.00 A.M. EACH DAY

DOG NO.	DAY	OCT IN μM $C^{14}O_2$ PER 0.5 ML SERUM	GOT (KARMEN UNITS)	GPT (KARMEN UNITS)
6	0	0.010	44	33
	1	0.238	150	45
	2	5.60	400	450
	3	10.2	520	1,250
	4	14.3	840	2,800
	6	13.5	400	4,000
	8	10.5	300	3,350
	10	8.40	200	3,350
	12	6.70	180	2,400
	14	8.60	170	2,400
8	0	0.045	42	30
	1	0.240	48	40
	2	4.00	110	110
	3	12.8	450	560
	4	23.0	680	1,100
	6	11.2	800	1,200
	8	10.5	580	*
	10	9.00	300	1,100
	12	8.60	250	1,250
	14	12.1	460	1,000
10	0	0.018	25	5
	1	5.50	650	380
	2	9.70	975	510
	3	18.8	1,075	1,100
	4	20.0	1,075	1,350
	6	13.8	750	1,500
	8	15.7	750	2,880
	10	14.3	850	3,200
	12	11.7	475	3,500
	14	6.70	305	2,000
7	0	0.020	42	18
	1	0.040	73	95
	2	0.072	104	62
	3	0.020	82	42
	4	0.014	37	42
	6	0.011	56	40
	8	0.015	42	24
9	0	0.011	35	12
	1	0.024	44	28
	2	0.010	32	15
	3	0.015	40	27
	5	0.009	50	28
	9	0.012	47	40
11	0	0.036	37	42
	1	0.570	70	180
	2	0.048	22	42
	3	0.043	32	95
	4	0.121	37	60
	6	0.065	25	55
	11	0.047	43	60

* Accidentally lost

TABLE V SERUM LEVELS OF OCT, GOT, AND GPT IN DOGS IN WHICH THE BILE FLOW WAS RESTORED AFTER CHOLEDOCHUS LIGATION DAYS ARE INDICATED WITH REFERENCE TO THE TIME OF THE FIRST OPERATION (DAY 0) THE SECOND OPERATION WAS PERFORMED BETWEEN 3 00 AND 9 00 P M BLOOD SAMPLES WERE TAKEN AT 10 00 A M THE DAY BEFORE AND ON EACH OF 3 DAYS AFTER OPERATION

DOG NO	DAY	OCT IN µM CHO PER 0 5 ML SERUM	GOT (KARMEN UNITS)	GPT (KARMEN UNITS)	REMARKS
6	14	8 60	170	2 400	
	15				Operation
	16	1 12	350	1 000	
	17	0 400	200	715	
	18				Dead
8	16	3 90	150	800	
	17				Operation
	18	2 60	180	1 440	
	19	0 250	20	500	
	20	0 070	26	580	
10	14	6 70	305	2 000	
	15				Operation
	16	1 92	120	1 500	
	17	2 28	120	1 050	
	18	2 11	130	930	

In 2 of the dogs all enzymes showed a reduction postoperatively, 1 dog (10 still had elevated OCT values, but the other serum enzymes tended to fall

Serum OCT in Carbon Tetrachloride Poisoning—The enzyme values ar set forth in Table VI and the mean for the 2 surviving dogs in Fig 3

In all animals that survived the maximum serum enzyme level was foun the second day OCT, GOT, and GPT reached respectively, 4 000 200 400 times the normal value Both OCT and GOT then fell rapidly GOT sh normal values on the tenth day but OCT was still slightly elevated after 14 when GPT was 10 times normal

DISCUSSION

Earlier it has been found* that in mammals OCT occurs only in the liver According to my results this is not altogether correct, in dogs very small amounts are found in the stomach, bile, and cecum and somewhat greater amounts in the small intestine Here, the content per unit weight is 1 5 per cent of that in the liver A similar OCT distribution occurs in human organs The discrepancy between my results and earlier ones is in all probability due to the more sensitive methods used in the present investigation

The important question arises as to whether extrahepatic OCT might influence its level in the serum, thus complicating the use of OCT in the diagnosis of liver disease However, the amounts in the stomach and cecum are so minute that they probably cannot influence the serum level appreciably Accordingly elevated OCT values are not found in peptic ulcer and ulcerative colitis In the small intestine the OCT content is low, although not negligible, but diseases of that organ are not common Therefore, elevated serum OCT values should point to liver damage if widespread disease of the small intestine can be ruled out

About 85 per cent of the OCT (liver extract) injected intravenously in healthy dogs was eliminated in the first 24 hours. Elimination of endogenous OCT proceeded at the same rate in the dogs with carbon tetrachloride poisoning (Table VI) and in one dog with ligated choledochus in which the bile flow was restored (No. 8, Table V).

TABLE VI. SERUM OCT, GOT, AND GPT VALUES AFTER CARBON TETRACHLORIDE POISONING. ONE MILLILITER CARBON TETRACHLORIDE PER KILOGRAM BODY WEIGHT WAS ADMINISTERED THROUGH A GASTRIC TUBE AT 11:00 A.M. ON DAY 0. BLOOD SAMPLES WERE TAKEN AT 10:00 A.M. EACH DAY

DOG NO.	DAY	OCT IN μM $C^{14}O_2$ PER 0.5 ML. SERUM	GOT	GPT	REMARKS
			(KARMEN UNITS)		
12	0	0.012	12	29	
	1	4.50	2,400	1,000	
	2	57.0	10,000	12,000	
	3	7.60	1,000	*	
	4	1.80	120	10,000	
	5	0.840	80	4,500	
	6	0.630	94	3,000	
	8	0.223	43	1,700	
	10	0.195	32	960	
	12	0.128	38	500	
	14	0.056	28	500	
13	0	0.018	24	30	
	1	12.0	4,200	4,600	
	2	62.0	6,750	11,000	
	3	2.40	850	3,000	
	4				Dead
14	0	0.030	25	32	
	1	1.24	400	1,100	
	2	92.0	3,700	9,500	
	3	41.0	240	9,600	
	4	3.70	80	3,900	
	5	1.28	77	2,900	
	6	1.04	56	1,860	
	8	0.320	47	800	
	10	0.095	24	400	
	12	0.090	18	300	
	14	0.110	25	245	
15	0	0.028	22	10	
	1	109.0	22,500	18,690	
	2				Dead

*Accidentally lost.

A similar rapid elimination of OCT also occurs in human beings, as shown in patients with gall stone colic, where the usual substantial rise of OCT subsides on the second day.[7]

Therefore elevated serum values indicate that biliary stasis or acute liver damage either is present or has been present and relieved not more than 2 days before the test.

After ligation of the common bile duct an initial delay of the OCT rise in serum appears. This delay is less marked when the gall bladder has been removed.[8] This different behavior is most readily explained by the pressure-equalizing effect of the gall bladder that is known to influence the serum bilirubin level in a similar way.

The dogs with carbon tetrachloride poisoning showed, on the second day, excessive serum enzyme concentrations, which for OCT amounted to 4,000 times the normal value After this huge OCT rise the serum values fell rapidly, thus suggesting that the damaged liver cells discharged their OCT content quickly

If the findings in dogs are applicable to man, determination of OCT could be useful in the diagnosis of liver disease In massive necrosis of the liver the serum OCT should rise quickly and then rapidly fall Total occlusion of the choledochus, on the other hand, should be associated with rising serum OCT values for a few days and then a gradual fall during the ensuing weeks

Clinical obstruction of the choledochus is often partial and few patients with this condition can be expected to show the OCT behavior typical for choledochus ligation in dogs Here, as in moderate and mild cases of hepatitis, further clinical studies in human subjects will have to decide if determination of serum OCT may be of diagnostic aid

SUMMARY

Ornithine carbamyl transferase (OCT) does not normally occur in the canine brain, lung, spleen, pancreas, kidney, striated muscle, or heart muscle Minutes amounts are found in bile, stomach, and large intestine The small intestine contains per unit weight about 1 5 per cent of the OCT content of the liver

In dogs, about 85 per cent of intravenously injected OCT disappears within one day and the rest within 6 days In choledochus ligation OCT rises rather slowly in serum the first day, then much faster, and reaches its peak on the fourth day with a serum level 1,000 times normal The OCT serum level is still 500 times normal after 14 days In severe carbon tetrachloride poisoning, maximum OCT values appear in serum on the second day, with 4 000 times normal Thereafter, the OCT level sinks rapidly and is only very slightly elevated after 14 days

I wish to thank Professor Henrik Lagerlof for helpful discussions and valuable criticism I am also indebted to Dr Åke Senning for help and advice in connection with the dog experiments

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TABLE I—CONT'D

PATIENT	AGE (YEARS)	DEGREE OF ATHERO-SCLEROSIS	DCA (MG./GM. OF BILE)	TCA (MG./GM. OF BILE)	DCA/TCA	DIAGNOSIS	NOTES
Children							
40	19	None	29.43	27.31	1.08	Posttraumatic edema of brain	Auto accident day of death
41	16	Slight	1.72	1.05	1.64	Atrial septal defect	Postoperative death hydronephrosis
42	14	None	13.11	6.90	1.90	Chronic glomerulonephritis	Hypertension, uremia
43	10	Slight	19.25	16.35	1.18	Cerebral trauma	Died 2 days after auto accident
44	2	None	0.72	0.83	0.87	Tuberculous meningitis	
45	4 mo.	None	1.97	4.83	0.41	Meningitis	
46	8 days	None	23.77	19.70	1.21	Tracheo-esophageal fistula	
47	5 days	None	7.91	16.68	0.47	Tracheo-esophageal fistula	
48	4 days	None	1.93	1.36	1.42	Hydronephrosis	Septicemia and deep jaundice
49	3 days	None	10.23	4.50	2.27	Prematurity	
50	2 days	None	4.30	1.98	2.17	Prematurity	
Averages	5.5		10.4	9.2	1.33		

THE INFLUENCE OF ALCOHOL AND DIETARY MAGNESIUM UPON HYPERCHOLESTEROLEMIA AND ATHEROGENESIS IN THE RAT

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MANY clinicians are proponents of the viewpoint that chronic alcoholic patients have less atherosclerosis than do nonalcoholic patients[1-5] Wilens[2] has suggested that this apparent difference may be explained in part by a decreased food intake, but to date only a few experiments have attempted to assess the role of alcohol in the pathogenesis of atherosclerosis,[6-8] and these studies have not shown a consistent relationship between alcohol and atherogenesis

Eberhard[7] demonstrated that alcohol markedly increased serum cholesterol levels in the rabbit This observation was not confirmed by Feller and Huff,[8] who reported that alcohol did not significantly alter serum cholesterol levels Furthermore, these authors were unable to demonstrate an effect of alcohol upon *atherogenesis in rabbits*

Flink[9] found decreased serum magnesium levels in chronic alcoholic patients while Klingman and associates[10] observed an increased tendency toward convulsions in rats fed alcohol and low magnesium diets These data suggested a relationship between alcohol and dietary magnesium Vitale and his coworkers[11] recently reported that rats fed a diet containing cholesterol and cholic acid developed vascular sudanophilia which was reduced significantly by increased levels of dietary magnesium

The present study investigated the interrelationships among these factors and the results showed that alcohol ingestion by rats increased vascular sudanophilia and serum cholesterol levels

METHODS

Weanling albino male rats* weighing 50 to 55 grams were fed a basic diet consisting of purified casein (10 per cent), glucose (58.1 per cent), salt mixture (5.0 per cent),† celluflour (5.0 per cent), choline chloride (0.3 per cent), vitamin ADE mixture

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†Salt mixture of Jones J H and Foster C J Nutrition 24 245 1942 with the $CaCO_3$ and $MgSO_4$ removed

(0 1 per cent),* $CaCO_3$ (1 5 per cent), and fat (Spry) (20 per cent) This diet was made atherogenic by the addition of 1 0 per cent of cholesterol and 0 3 per cent cholic acid with a proportionate reduction in the amount of glucose necessary to keep constant the percentage of protein and fat in the solid diet The animals were housed in individual cages and fed ad libitum Groups of animals were fed either the basic diet or the diet containing cholesterol and cholic acid These two groups were further subdivided into those receiving either water or 20 per cent alcohol as the only source of fluid Magnesium as magnesium oxide was added in concentrations varying from 6 to 192 mg per 100 grams of diet

Food alcohol, and water intakes were measured daily Animals were weighed biweekly, and observed for signs of magnesium deficiency, e g , hyperexcitability and hyperemia of the ears, feet, and tail

Animals were sacrificed at the end of 28 or 29 days Serum cholesterol levels were determined according to the method of Carpenter and associates[10] Sections of the gastrointestinal tract, kidney, liver, and heart were fixed in 10 per cent formalin for microscopic examination The left kidney was decapsulated and weighed Hearts were removed with the aorta attached and opened in a manner to expose the aortic and mitral valves After fixation in formalin these specimens were stained with Sudan IV The aortic and mitral valves, the intervalvular endocardium and the aorta were examined by use of a dissecting microscope The sudanophilia was estimated on a 0 to 4 plus scale by two of the authors working independently and the total vascular sudanophilia was designated as "heart score"[11, 13] The degree of calcium deposition in the renal tubules was evaluated in the manner previously described and designated as "kidney score"[13]

RESULTS

Table I shows that the serum cholesterol levels of animals drinking 20 per cent alcohol were significantly higher than those of animals fed only water There was no apparent correlation between the level of dietary magnesium and serum cholesterol in animals fed alcohol or water

Heart scores were 7, 9, and 3 for the groups fed 20 per cent alcohol and the 24, 96, and 192 mg per cent magnesium diets, respectively The daily average food intake for these 3 groups was approximately 7 0 Gm The heart scores of animals fed water were 6, 5, and 4, and they ingested an average of 9 8 Gm per day over the same period

As was expected, high levels of dietary magnesium reduced the heart score of rats fed only water from 6 at 24 mg per cent to 4 at 192 mg per cent Although those animals which were fed alcohol had significantly higher serum levels of cholesterol the amount of vascular sudanophilia seen was significantly increased in only one instance The group fed a diet containing 96 mg per cent of magnesium and alcohol had a heart score of 9 while those fed the same level of dietary magnesium with water had a heart score of only 5

The addition of alcohol resulted in decreased food intake as well as decreased total caloric intake† Similarly, the decreased caloric intake was accompanied by a decreased rate of weight gain The level of dietary magnesium was without effect on weight gain in both the alcohol and water fed groups

The kidney "profile' was evaluated by (1) gross appearance, (2) the weight of the kidney, (3) the ratio of kidney weight to body weight (K/B ratio), and

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*A mixture of vitamins A D and E was prepared by adding 5 Gm of halibut oil and 1 Gm of alpha tocopherol acetate to 44 Gm of corn oil (Mazola) The following vitamins were added per kilogram of diet 4 mg thiamine hydrochloride 8 mg riboflavin 4 mg of pyridoxine hydrochloride 25 mg of calcium pantothenate and 40 mg of niacin

†The caloric value of alcohol was calculated as 7 calories per gram of absolute alcohol

TABLE II. THE EFFECT OF 20 PER CENT ALCOHOL ON SERUM CHOLESTEROL, HEART SCORE, KIDNEY PROFILE, AND GROWTH OF RATS FED A DIET CONTAINING CHOLESTEROL (1 PER CENT) AND CHOLIC ACID (0.3 PER CENT) (EXPERIMENT II)

DIETARY MAGNESIUM (MG. PER CENT)	ALCOHOL	SERUM CHOLESTEROL (MG. PER CENT)	HEART SCORE	KIDNEY PROFILE			WEIGHT GAIN (GM.)	AVERAGE FOOD INTAKE PER DAY (GM.)	AVERAGE LIQUID INTAKE PER DAY (ML.)	AVERAGE CALORIES PER DAY
				KIDNEY SCORE	KIDNEY WEIGHT (MG.)	K/B RATIO				
6	-(8)*	762	5.3	3	572	0.69	23	7.4	11.3	35.5
12	-(8)	645	5.6	3	624	0.61	41	9.2	12.7	44.2
	+(5)	784	6.9	2	440	0.56	19	6.1	6.2	36.1
24	-(8)	601	3.2	1	463	0.45	44	10.1	11.1	48.5
	+(6)	1,395	4.4	1	401	0.50	21	6.2	6.2	36.6
96	-(8)	597	1.9	1	462	0.45	42	9.6	10.4	46.1
	-(8)	1,145	4.0	0	408	0.52	18	6.3	5.9	36.7
192	-(8)	653	2.1	0	447	0.46	38	9.3	10.1	44.6
	+(7)	1,331	2.5	0	373	0.57	7	5.8	6.0	34.4
Correlation coefficient	–		-0.45 (P<0.01)			-0.47 (P<0.01)				
	+		-0.61 (P<0.01)			+0.15				

*Number of animals in each group

TABLE III. THE EFFECT OF 20 PER CENT ALCOHOL ON SERUM CHOLESTEROL, KIDNEY PROFILE, AND GROWTH OF RATS FED A DIET FREE OF CHOLESTEROL AND CHOLIC ACID (EXPERIMENT II)

DIETARY MAGNESIUM (MG. PER CENT)	ALCOHOL	SERUM CHOLESTEROL (MG. PER CENT)	KIDNEY PROFILE			WEIGHT GAIN (GM.)	AVERAGE FOOD INTAKE PER DAY (GM.)	AVERAGE LIQUID INTAKE PER DAY (GM.)	AVERAGE CALORIES PER DAY (ML.)
			KIDNEY SCORE	KIDNEY WEIGHT (MG.)	K/B RATIO				
6	– (8)*	82	3	622	0.62	41	9.4	11.9	45.1
12	– (8)	90	3	679	0.59	54	11.5	12.2	55.2
	+ (4)	107	3	467	0.62	15	6.2	5.6	36.0
24	– (8)	88	1	490	0.45	50	9.9	11.8	47.5
	+ (6)	115	1	425	0.53	20	5.6	6.1	33.6
96	– (8)	76	0	476	0.40	58	10.1	11.9	48.5
	+ (7)	104	0	468	0.53	29	7.6	8.4	45.7
192	– (8)	75	0	482	0.40	59	10.5	11.1	50.4
	+ (7)	92	0	442	0.47	34	6.4	7.3	38.7
Correlation coefficient	–				-0.57 (P<0.01)				
	+				-0.79 (P<0.01)				

*Number of animals in each group

(4) the kidney score. The effect of magnesium deficiency upon the kidneys was similar to changes previously described.[1, 2] On gross examination kidneys showing the "magnesium effect" were usually large and pale, and the severe cases revealed surface speckling subsequently identified as tubular dilatation. Microscopic examination revealed the deposition of laminated calcium stones within the lumina of the collecting tubules of the outer third of the zona intermedia. We have called this change renal tubular microlithiasis. Occasionally the microliths were found in the medullary tips, and their presence was not correlated with the severity of the deposits in the remainder of the medulla. Other microscopic findings included dilatation of the tubules surrounding the microliths with flattening of the cuboidal epithelium, focal epithelial hyperplasia, and local giant cell formation as well as slight to moderate dilatation of the proximal nephron. Focal interstitial infiltrates of lymphocytes and plasma cells were also noted in the cortex and medulla.

Alcohol ingestion completely protected against renal tubular microlithiasis in this experiment (Table I). The kidney score of animals fed only water was 1 at 24 mg. per cent and was decreased to a score of 0 at the highest level of dietary magnesium. The average weight of the kidneys of the alcohol fed animals was slightly less than that of animals on water. The K/B ratio was reduced from 0.52 at 24 mg. per cent to 0.42 at 192 mg. per cent in animals fed water. However, the K/B ratio was always higher in alcohol fed animals.

In Experiment II (Table II) two lower levels of dietary magnesium were added. Animals fed 6 mg. per cent of magnesium were not given alcohol since in our experience these animals have not survived such a regimen. Animals fed alcohol instead of water had significantly higher levels of serum cholesterol despite the fact that they ingested approximately 35 per cent less food. The average serum cholesterol level for animals ingesting water varied only from 596 to 762 mg. per cent regardless of the level of dietary magnesium. Alcohol fed animals had serum cholesterol levels ranging from 800 to approximately 1,400 mg. per cent.

The heart scores of animals fed only water were 5.3 at 6 mg. per cent magnesium, 5.6 at 12 mg. per cent, 3.2 at 24 mg. per cent and approximately 2 at 96 and 192 mg. per cent. Heart scores of animals fed alcohol, however, were 6.9 at 12 mg. per cent magnesium, 4.4 at 24 mg. per cent, 4.0 at 96 mg. per cent, and 2.1 at 192 mg. per cent. The correlation coefficient calculated for dietary magnesium versus heart score was 0.45 for the group fed water and 0.61 for the group fed alcohol, both of which are significant at the 0.01 level ($P<0.01$).

When cholesterol and cholic acid were omitted from the diet (Table III) the average serum cholesterol level (103 mg. per cent) of the alcohol fed group was slightly higher than that of the water fed animals (82 mg. per cent). Dietary magnesium had no effect on serum cholesterol levels in either group. The heart scores, as expected, in this group were zero.

Maximal weight gain of alcohol fed animals occurred at dietary magnesium levels of 96 to 192 mg. per cent while water fed animals grew maximally at 12 mg. per cent.

TABLE IV. EFFECT OF 20 PER CENT ALCOHOL AND MAGNESIUM ON GASTROINTESTINAL HEMORRHAGE

MAGNESIUM (MG. PER CENT)	ATHEROGENIC DIET	NONATHEROGENIC DIET
12	3/8*	4/8
24	2/8	2/8
96	0/8	1/8
192	1/8	1/8

*Number of animals dying with GI hemorrhage.

In contrast to Experiment I, alcohol did not prevent renal tubular microlithiasis. The addition of magnesium to the nonatherogenic diet with and without alcohol and to the atherogenic diet without alcohol significantly reduced the K/B ratio but did not reduce the elevated ratio in animals fed the atherogenic diet and alcohol (Tables I, II, and III). In general, the atherogenic diet alone caused greater K/B ratios than did the nonatherogenic diet. However, even when large amounts of magnesium were added to the atherogenic diet the K/B ratio remained elevated. It was also noted that alcohol tended to increase the K/B ratios of animals fed the atherogenic and nonatherogenic diets.

Fourteen alcohol-fed rats in Experiment II died with moderate to marked gastrointestinal hemorrhage. The largest number of deaths occurred in those rats fed the low-magnesium diets (Table IV). There was no significant difference between the groups fed either the atherogenic or nonatherogenic diet. The stomachs and intestines were partially to completely filled with changed black or brown blood. On gross examination, a few stomachs were focally congested and showed petechial-like hemorrhages in the glandular portion. No ulcerations were found in either stomach or duodenum. Microscopic examination of the stomach and duodenum revealed only slight to moderate autolysis of the glandular portion of the mucosa.

DISCUSSION

Weanling rats fed the cholesterol-cholate diets regularly developed hypercholesterolemia. The addition of 20 per cent alcohol to this diet caused as much as a twofold increase in the degree of hypercholesterolemia, although the food intake was approximately one-third less. This is essentially in agreement with the studies of Eberhard[6] who noted elevated serum cholesterol levels in alcohol-fed rabbits on an atherogenic diet.

The cause of the elevated serum cholesterol levels associated with alcohol intake is not clear. Whether alcohol facilitates the absorption of cholesterol, or promotes endogenous synthesis of cholesterol, or impedes the degradation of endogenously produced cholesterol are questions which remain to be investigated.

On gross examination, livers of both the alcohol and water-fed animals on the atherogenic diet were enlarged and pale. Microscopic examination revealed slight fatty vacuolization of hepatic cells in both groups of animals. Thus, it was not possible to correlate the variations in serum cholesterol levels with the liver morphology.

Animals ingesting alcohol did not consume a 10 per cent protein diet. Since the amount of alcohol ingested comprised part of the total caloric intake, the proved. Furthermore, the alleged reduction in atherosclerosis attributed to alcoholism may indeed be due to the effect of alcohol upon the dietary habits of alcoholics, who in general, eat fewer calories, fat, and protein during periods of intensive drinking. Further clinical studies correlating, at least, dietary habits, serum cholesterol, and magnesium levels with the degree of atherosclerosis are indicated before one can relate alcohol ingestion per se to atherogenesis.

The apparent contradiction between the results reported here and those of Eberhard[6] and more recently Nichols and associates[7] who found that alcohol did not promote the development of atherogenesis in rabbits and chickens may be partially explained by the higher levels of protein used by these investigators. It seems likely that the atherogenic effect of alcohol is mediated in part by an increased magnesium requirement. This effect is apparent only at low levels of protein. Vitale and co-workers[11] demonstrated that high dietary magnesium did not exhibit any protective effect on heart sudanophilia when 20 per cent protein was incorporated into diets containing 1 per cent cholesterol and 0.3 per cent cholate. Thus, a diet high in protein may mask the effect of alcohol in increasing the magnesium requirement.

The results of these studies and others[23] raised the question as to whether or not cholesterol and cholic acid aggravated the kidney lesions attributed to low-magnesium diets. It is not possible to distinguish the kidney of an animal fed an atherogenic diet from that of an animal fed a nonatherogenic diet by weight alone. In experiments reported by Hellerstein and associates[13] feeding cholesterol and cholic acid usually increased the amount of renal tubular microlithiasis and, indeed, tubular calcification has been used as an indication of magnesium deficiency. In the present study, this finding was not constant. On the other hand, the ratio of kidney weight to body weight was always elevated in animals fed cholesterol and cholic acid, and it appeared that the gross morphology, coupled with the kidney to body weight ratios, was a more consistent index of magnesium deficiency in cholesterol and cholic acid-fed animals than was renal tubular calcification. In subsequent studies, Vitale and associates[21] also made these observations.

Gastrointestinal hemorrhage was noted in 14 rats which died prior to the termination of Experiment II. All 14 animals were on 20 per cent alcohol and there was no significant difference between the groups fed atherogenic and nonatherogenic diets. Increased levels of dietary magnesium protected the rat against gastrointestinal hemorrhage. Unfortunately, postmortem autolysis interfered with the histologic study of the upper gastrointestinal tract and may have masked gastric lesions.

SUMMARY

Weanling male rats were fed diets made atherogenic by the addition of 1 per cent cholesterol and 0.3 per cent cholic acid. One group of animals received 20 per cent alcohol as the only source of fluid and another group received water. Magnesium was added in the form of magnesium oxide in varying concentrations from 6 to 192 mg. per 100 Gm. of diet.

The results of this study demonstrated that rats fed an atherogenic diet and 20 per cent alcohol ate approximately 35 per cent less food but had higher

serum cholesterol levels and more extensive vascular sudanophilia than rats fed an atherogenic diet with water High levels of dietary magnesium lowered the amount of lipid deposition

It was also shown by the kidney profile and weight gain that alcohol increased the magnesium requirements of animals fed the basic diet, and that the addition of cholesterol and cholic acid accentuated the deficiency Possible mechanisms for these observations were discussed

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SERUM CHOLESTEROL LEVELS IN ATHEROSCLEROTIC PATIENTS TREATED WITH ORAL ANTICOAGULANTS

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Anticoagulant therapy has proved to be of value in the treatment of the thrombotic complications of coronary atherosclerosis. However, other possible actions of the oral anticoagulants have not been intensively studied. The direct-acting anticoagulant, heparin, has antilipemic properties and prolonged heparin treatment of patients with myocardial infarction has been claimed by Engelberg[1] to result in a lowered mortality and morbidity in such patients. This may be associated with the antilipemic properties of heparin.

Although the oral anticoagulants have a different mechanism of action than heparin and need not necessarily have actions similar to heparin on serum lipids, nevertheless it seemed of interest to study the effect of these agents on serum lipids of subjects who had been placed on these drugs because of thrombotic episodes or evidence of impending thrombosis.

METHODS

Five groups of patients were studied. The first group consisted of 8 individuals treated with phenylindanedione; the second consisted of 10 subjects who received acenocoumarin; and a third group was composed of 6 patients who were given ethyl biscoumacetate.* The age range in each of these 3 groups was from 50 to 70 years. All these patients had evidence of impending myocardial infarction or had experienced a recent myocardial infarction; these two types were distributed equally throughout the three groups.

Since many of the atherosclerotic subjects in all groups had shown evidence of vascular disease, the majority had been placed on diets of moderate fat and caloric intake months or weeks previous to the study. The rigidity of adherence to these diets is not known, but the diets of all patients, as far as could be ascertained, remained the same during the periods of observation.

The anticoagulants were administered orally in divided doses daily in amounts sufficient to maintain the prothrombin time, estimated by the one-stage method of Quick, between 25 and 30 per cent of normal. Blood samples were taken at intervals over the first 10 weeks of therapy and again over a period between 24 and 32 weeks after starting therapy.

Another group, consisting of 7 middle-aged atherosclerotic subjects, receiving no anticoagulant therapy, were observed over a 6-week period. A fifth group of 5 atherosclerotic individuals were treated with heparin in doses insufficient to produce anticoagulation.

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*Acenocoumarin is marketed as Sintrom, ethyl biscoumacetate as Tromexan. Both preparations were supplied by Geigy Pharmaceuticals, Montreal.

In the latter group all subjects had blood samples taken before starting treatment. They were then given 200 mg. of heparin subcutaneously every 48 hours over a 6 week period, blood samples were taken 2 days after the first dose of heparin and thereafter at approximately weekly intervals 48 hours after an injection of heparin and just prior to the next injection.

The blood samples were centrifuged and the separated serum either analyzed the same day or quickly frozen and stored at -15° C. and analyzed later. The following determinations were made on the sera of the patients: total serum cholesterol by the method of Pearson and associates[2], total serum lipids using the turbidimetric method of Kunkel and co-workers[3], and serum lipoprotein was determined by paper electrophoresis after the method of Jencks and associates.[4] The separated lipoproteins were stained with oil red O. The stained lipoproteins were eluted with methanolic chloroform and the optical density measured in a Beckman model B spectrophotometer. The β lipoprotein was expressed as a per cent of the sum of the optical densities of the α plus β lipoprotein lipid. Duplicate determinations agreed within 7 per cent. Lipoprotein determinations made on the 24 to 32 week samples were measured with an automatic scanning device (Spinco Analytrol). Results obtained by scanning and eluting the same strips were similar.

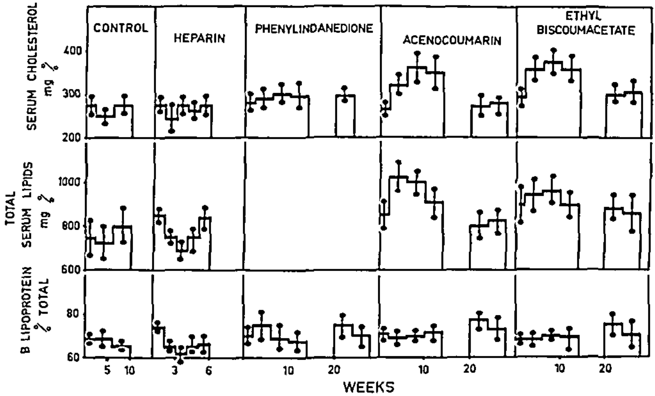

Fig. 1.—The serum cholesterol, total serum lipids, and lipoprotein levels in atherosclerotic patients receiving anticoagulants.

RESULTS

It will be seen from Fig. 1 that significant ($p < 0.05$) elevations occurred following the initiation of treatment with the coumarin derivatives, acenocoumarin and ethyl biscoumacetate. However, when investigated again after 24 to 32 weeks, the serum cholesterol in these patients had returned to the levels found at the beginning of anticoagulant therapy. This rise following treatment was not apparent in the similar patients who received phenylindanedione. The control group of untreated atherosclerotic patients showed no significant variation in the serum cholesterol over a 10 week period of study. In the heparin treated group no significant alterations in cholesterol were found over the 6 week period this therapy was carried on.

The total serum lipids tended to parallel the serum cholesterol levels; significant elevations were noted following acenocoumarin and ethyl biscoumacetate therapy, but no real changes occurred in the untreated group. In the heparin-treated group the total serum lipids fell significantly from the pretreatment levels but this decrease was not maintained.

No significant changes were observed to occur in the β-lipoprotein measured as a per cent of the total lipoprotein except in the subjects treated with heparin. In this group there was an early fall in the proportion of β-lipoprotein which was maintained for 2 weeks and thereafter tended to return to pretreatment values in some of the subjects.

DISCUSSION

There is an apparent difference between the serum cholesterol of the groups treated with acenocoumarin and ethyl biscoumacetate and the group treated with phenylindanedione. In the acenocoumarin and ethyl biscoumacetate groups, significant elevations in serum cholesterol occurred but these did not appear to be maintained. No changes were apparent in similar patients treated with phenylindanedione. Bernard-Griffiths[5] has reported that a combination of small doses of Dicumarol and choline given to atherosclerotic individuals produced a slight increase in serum cholesterol. Although not strictly comparable with our studies, this report and our findings with the coumarin derivatives are in accord. Reiniš and co-workers[6] have reported that administration of ethyl biscoumacetate to cholesterol-fed rabbits accelerates the rate of increase in serum cholesterol and rate of evolution of atheromatous lesions which usually follow cholesterol feeding in the rabbit.

There are several possible explanations for the difference in the serum cholesterol values of the patients treated with the coumarin derivatives. The coumarins may have brought about a decreased catabolism or an increased synthesis or absorption of cholesterol, or they may have caused a mobilization of cholesterol from vascular plaques. Whatever the mechanism, it does not appear to be persistent, for by the twentieth week of treatment the cholesterol level was back to pretreatment values.

The lack of response of serum cholesterol in the heparin-treated group is in accord with the findings of others.[7, 8] However, the total serum lipids and the β-lipoprotein fell in these individuals. These results are in agreement with those of Soffer[7] who reported a fall in total serum lipids in subjects treated with heparin. These changes may be in some way related to the clearing of an alimentary lipemia which follows heparin injection. In the present series, all subjects showed initial changes in total serum lipids and lipoproteins after heparin injections were started. However, in 3 subjects these changes were not maintained and the lipid levels and β-lipoprotein were observed to have returned to pretreatment levels in the blood samples taken 4 weeks after the institution of treatment.

The findings reported here indicate that dicoumarin derivatives, particularly acenocoumarin, temporarily elevate the serum cholesterol and total serum lipids.

Whether or not this is of any importance in the use of those agents as anticoagulants in atherosclerotic patients remains to be determined

SUMMARY

Atherosclerotic subjects were treated with heparin or the oral anticoagulants, phenylindanedione, or dicoumarin derivatives (acenocoumarin and ethyl biscoumacetate), and the serum cholesterol, total serum lipids, and β lipoproteins were measured

In the phenylindanedione treated group no significant change occurred in any of these serum lipids In patients treated with acenocoumarin and ethyl biscoumacetate, elevations occurred in the serum cholesterol and total serum lipids which were maintained for at least 10 weeks but were back to pretreatment levels after 24 to 32 weeks of therapy

In none of the groups treated with oral anticoagulants were any significant alterations in the β lipoproteins noted

In the group treated with heparin no change was noted in the serum cholesterol levels but significant falls were found in the levels of total serum lipids and beta lipoproteins

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STUDIES ON ERYTHROPOIESIS

X. The Use of Bone Marrow Tissue Culture in Demonstrating Erythropoietin

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IN RECENT years, the kinetics and controlling mechanisms of erythrocyte production have been studied extensively. One of the most important and interesting results of these investigations is the demonstration that a humoral substance(s) (erythropoietin) is responsible, at least in part, for the increased erythropoiesis in response to hemorrhage,[1-4] hemolytic anemia,[5-6] hypoxia,[7-8] and cobalt administration.[9] Furthermore, indirect evidence has been accumulated showing that the amount of this factor(s) is decreased when the production of erythrocytes is reduced as in transfusion-induced polycythemia[10] and hyperoxia.[11]

To date, nearly all assays of erythropoietic activity have been carried out in whole animals. Increases in reticulocyte, hemoglobin, and hematocrit values,[2-7] as well as the increase in radioiron uptake,[1, 8] have been used as parameters in assessing the effect of erythropoietin-rich serum on normal, starved hypophysectomized, or polycythemic animals. Qualitative estimates of erythropoietic activity in the spleen and bone marrow have also been used as evidence of the presence or absence of erythropoietin.[10] However, only one report[12] of the quantitative estimation of the effect of serum rich in erythropoietin on proliferation of erythrocyte precursors was found in the literature.

The improvement in tissue culture techniques has made it possible to maintain tissue cells in vitro. It is the purpose of this paper to present some experiments wherein the effect of different sera upon bone marrow cells grown in tissue culture is measured quantitatively.

MATERIALS AND METHODS

The chambers used in these experiments for the cultivation of the marrow cells were a modified version of the Mackaness and Smith monocyte culture chamber† (Fig. 1, *A* and *B*).[13] This consists of a block of unplasticized Perspex 4.9 by 3.2 by 0.22 cm. In the center, circular holes with a diameter of 23 mm. are counterbored from each side to within 1.5 mm. of each other, and a hole, 19 mm. in diameter, is drilled completely through. Thus the central hole is surrounded by a recessed border on both sides. From the side, two small holes, 0.7 mm. in diameter, are drilled into the central hole. The plug consists of two parallel

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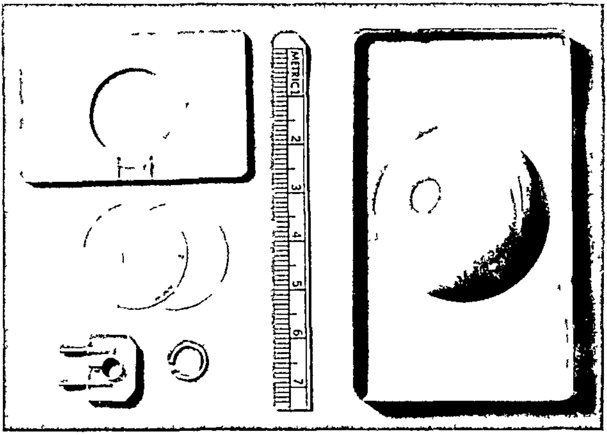
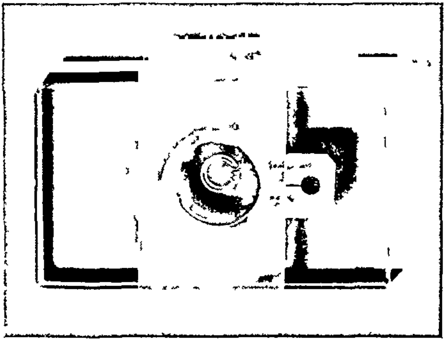
†Obtained from H. G. East & Company, Oxford, England, and constructed in the Engineering Division of the Argonne Cancer Research Hospital, Chicago, Ill.

wires of slightly smaller diameter set in a small piece of plastic at the same distance apart as the two lateral holes A Perspex ring, 1 mm high and 6 mm inside and 8 mm outside diameters, from which a small arc has been removed completes the apparatus

A





B

parts of the modified Mackaness and Smith monocyte culture
Upper left, main block middle left cover glasses lower left
ht inverted shallow well glass slide used to support main block
odified Mackaness and Smith monocyte culture chamber fully

The plastic chamber is constructed after sterilization in dry heat (100 to 105° C) for 1 hour, using paraffin (melting point 56 to 58° C) A chemically clean and sterile circular cover slip, 22 mm in diameter, is placed on one recessed border and secured with melted wax The chamber is inverted after cooling, and the previously waxed central ring is placed so that a needle may be inserted through a lateral hole and into the center of the ring through the missing arc. A cover glass is placed on the top recessed edge, and the bottom cover glass is gently heated to melt the wax on the central ring, thus securing it. The top glass is then secured with melted wax.

The culture medium used was Earle's salt solution* with added vitamins,* amino acids,* and glutamine* in amounts twice Eagle's specifications.[14-16] The serum used was taken under sterile conditions by cardiac puncture from Holtzman strain rats that had been fasted overnight. The following sera were tested: (1) serum from normal rats weighing 200 to 300 grams (normal serum); (2) serum from rats that had been made anemic by hemorrhage on two consecutive previous days and in which the hematocrit was less than 25 per cent (anemic serum); (3) serum from rats given cobaltous chloride (25 μM per 100 Gm. body wt.) with tracer amounts of radioactive cobalt^{60} ten hours previously (cobalt serum); (4) normal rat serum with an amount of cobaltous chloride added in vitro equal to the amount present in the cobalt serum (cobalt control serum); and (5) serum from rats that had been rendered polycythemic by tranfusion of erythrocytes equal to 4 per cent of the body weight given over a period of 3 days and in which the hematocrit was 65 to 70 per cent (polycythemic serum).

The marrow cells were obtained by means of sterile dissection of both femurs of a rat killed by cervical fracture and were placed in 5 ml. of Eagle's salt solution to which Versenate (disodium ethylenediaminetetraacetate) had been added in a concentration of 1.5 mg. per milliliter. The cells were agitated, and the multicellular clumps allowed to settle to the bottom by tilting the tube at an angle of 45 degrees in ice water for 5 to 10 minutes. The supernatant suspension of single cells and smaller clumps of cells was removed and centrifuged for 10 minutes at 1,200 r.p.m.; the acellular supernatant was removed. The cells were resuspended in enough Eagle's medium (without serum) to make a concentration of 6,000 to 8,000 nucleated cells per cubic millimeter. Two parts of this cellular suspension were added to 1 part of each of the sera being tested. Likewise, 2 parts of Eagle's medium were added to 1 part of each of the sera, making the medium complete.

Inside the ring of each chamber was placed 0.06 ml. of the cellular suspension by means of a tuberculine syringe equipped with a No. 22 needle. The space between the ring and the main part of the chamber was partially filled with the corresponding complete medium, leaving an air bubble. The plug was dipped in a 1:1 mixture of paraffin and yellow petrolatum, and the wires were inserted into the 2 lateral holes. The chambers were incubated at 37° C. and rotated at intervals of 6 to 12 hours. Ten to fifteen replicate cultures were prepared for each serum tested.

At specified time intervals, the upper cover glass was removed and the ring loosened. The medium was agitated gently to remove all cells from the bottom and was carefully aspirated and measured with a tuberculin syringe graduated in 0.01 ml. units. The chamber was washed twice with 0.2 ml. of 2 per cent acetic acid, and the recovered wash fluid was measured. No cells remained on the bottom cover glass. The number of nucleated cells per cubic millimeter was counted without further dilution on a regular Neubauer hemocytometer; two separate aliquots were used. The total number of cells removed was calculated. This process was performed on 3 chambers of a particular serum at each time.

Slides for differential count were made on 2 chambers of each serum at each time. The top cover glass was detached, and the supernatant fluid was removed carefully while the central ring was still attached. The cells remaining on the bottom cover glass were quickly air-dried and stained with Wright's stain after removing the cover glass from the chamber and the ring from the cover glass. The cover glasses were mounted, cells downward, on regular glass slides and labeled so that they could be counted as unknowns. Differential count of 1,000 cells on each slide was performed in a systematic fashion, counting the cells in separate microscope fields along the horizontal, vertical, and (occasionally) diagonal diameters of the cellular area.

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*Obtained from Microbiological Supplies, Bethesda, Md.

From these data, the total number of any particular cell type or types can be calculated

$$\text{Number of type A cells} = (\text{total cells per mm}^3 \times \text{mm}^3) \times \frac{\%\ \text{of type A cells}}{100}$$

RESULTS

The number of erythrocytic precursors was plotted against time of incubation (see Figs 2, 3, and 4) In the presence of normal serum the number of these cells decreased linearly throughout the 24 hour period In cultures incubated with anemic serum, the number of these cells increased during the first 12 hours and decreased during the second 12 hours Cobalt serum elicited the same response, and cultures incubated in cobalt control serum behaved similar to those in normal serum With polycythemic serum, the number of erythrocyte precursors at 12 hours was significantly below the number in cultures using normal serum During the following 24 hours (12 to 36 hour incubation) the number of erythrocyte precursors in the polycythemic cultures was lower than in the normal cultures but the difference was not so striking

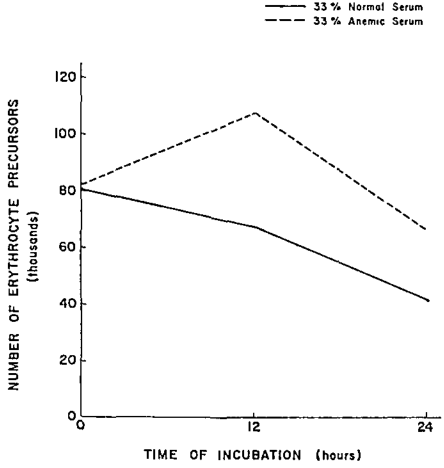

Fig 2—Total number of erythrocyte precursors in bone marrow tissue cultures grown in normal and anemic sera

The number of each of the erythrocyte precursors (rubriblast, prorubricyte, rubricyte, and metarubricyte) was analyzed for each of the time intervals (Tables I, II, and III) In 3 out of the 4 experiments (anemic or cobalt serum), it was observed that at 12 hours the number of metarubricytes was increased above the original number at 0 hour, this increase was maintained

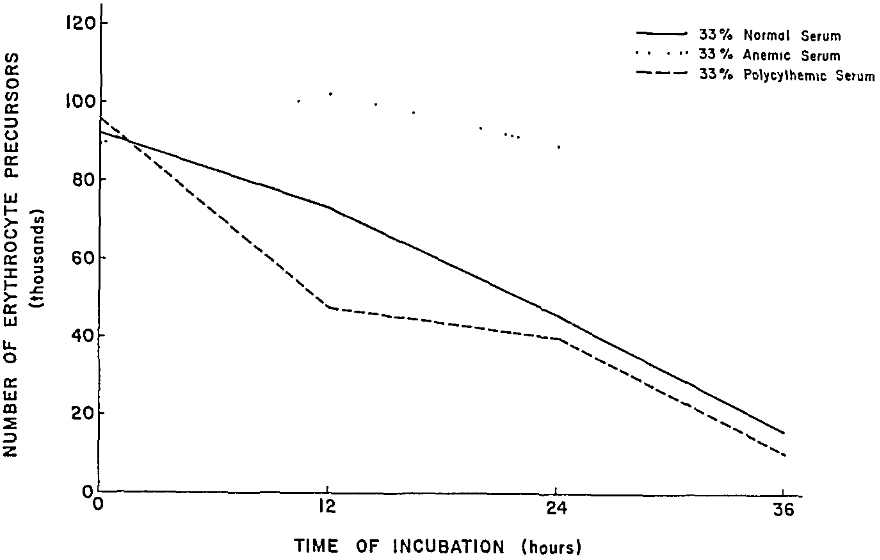

Fig. 3.—Total number of erythrocyte precursors in bone marrow tissue cultures grown in normal, anemic, and polycythemic sera.

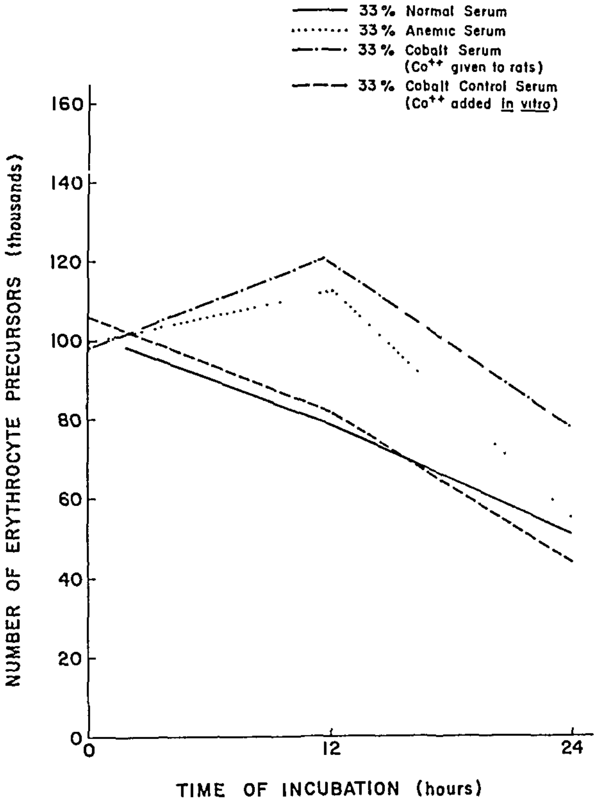

Fig. 4.—Total number of erythrocyte precursors in bone marrow tissue cultures grown in normal, anemic, cobalt, and cobalt control sera.

through 24 hours. In one experiment with anemic serum (Experiment 3), the number of metarubricytes was only slightly higher at 12 hours and had decreased by 24 hours. At this same interval the number of rubricytes and prorubricytes was increased over the original number and was considerably higher than the number of these cells in normal cultures. During the second 12 hour period, the number of these cells decreased at a rate greater than that seen in normal cultures. Rubriblasts are so scarce in these cultures that changes in their number cannot be accurately assessed.

TABLE I (EXPERIMENT 1). NUMBERS AND PERCENTAGES (OF TOTAL NUCLEATED CELLS) OF ERYTHROCYTE PRECURSORS IN BONE MARROW CULTURED WITH NORMAL AND ANEMIC SERA AFTER VARYING INTERVALS OF INCUBATION

		12 HOURS		24 HOURS	
PRECURSOR	0 HOUR	NORMAL	ANEMIC	NORMAL	ANEMIC
Total cell count	301,240	267,430	271,170	199,630	245,480
Metarubricyte					
Number	26,810	44,130	45,010	30,540	43,690
Per cent	8.9	16.5	16.6	15.3	17.8
Rubricyte					
Number	28,610	17,380	37,690	8,980	15,710
Per cent	9.5	6.5	13.9	4.5	6.4
Prorubricyte					
Number	24,010	5,350	21,960	1,200	5,150
Per cent	8.0	2.0	8.1	0.6	2.1
Rubriblast					
Number	2,410	2,670	2,980	200	490
Per cent	0.8	0.1	1.1	0.1	0.2
Total erythrocyte precursors					
Number	79,430	69,530	107,640	40,920	65,040
Per cent	27.2	25.1	39.7	20.5	26.5

TABLE II (EXPERIMENT 2). NUMBERS AND PERCENTAGES (OF TOTAL NUCLEATED CELLS) OF ERYTHROCYTE PRECURSORS IN BONE MARROW CULTURED IN NORMAL, ANEMIC, AND POLYCYTHEMIC SERA AFTER VARYING INTERVALS OF INCUBATION

	12 HOURS			24 HOURS			36 HOURS	
PRECURSOR	NORMAL	ANEMIC	POLYCYTHEMIC	NORMAL	ANEMIC	POLYCYTHEMIC	NORMAL	POLYCYTHEMIC
Total cell count	252,380	260,050	228,320	208,310	252,680	187,160	143,290	135,100
Metarubricytes								
Number	40,380	64,490	31,050	31,250	65,410	26,760	10,750	6,620
Per cent	16.0	24.8	13.6	15.0	25.9	14.3	7.5	4.9
Rubricytes								
Number	22,460	26,780	9,580	10,830	17,180	10,290	4,300	2,560
Per cent	8.9	10.3	4.2	5.2	6.8	5.5	3.0	1.9
Prorubricytes								
Number	9,340	8,580	7,300	4,170	5,550	2,990	500	800
Per cent	3.7	3.3	3.2	2.0	2.2	1.6	0.4	0.6
Rubriblasts								
Number	500	2,080	200	0	700	200	200	100
Per cent	0.2	0.8	0.1	0.0	0.3	0.1	0.2	0.1
Total erythrocyte precursors								
Number	72,680	101,930	48,130	46,250	88,840	40,240	15,750	10,080
Per cent	28.8	39.2	21.1	22.2	35.2	21.5	11.1	7.5

TABLE III. (EXPERIMENT 3) NUMBERS AND PERCENTAGES (OF TOTAL NUCLEATED CELLS) OF ERYTHROCYTE PRECURSORS IN BONE MARROW CULTURED IN NORMAL, ANEMIC, COBALT CONTROL, AND COBALT SERA AFTER VARYING INTERVALS OF INCUBATION

PRECURSOR	0 HOUR	12 HOURS				24 HOURS			
		NORMAL	ANEMIC	COBALT CONTROL	COBALT	NORMAL	ANEMIC	COBALT CONTROL	COBALT
Total cell count	232,990	245,420	217,230	249,420	246,560	184,620	202,030	155,790	229,790
Metarubricytes									
Number	58,010	49,080	59,030	55,120	67,800	38,400	33,130	35,050	69,170
Per cent	24.9	20.0	27.3	22.1	27.5	20.8	16.4	22.5	30.1
Rubricytes									
Number	27,260	21,840	33,020	21,700	25,400	9,800	11,920	7,480	10,340
Per cent	11.7	8.9	15.2	8.7	10.3	5.3	5.9	4.8	4.5
Prorubricytes									
Number	14,450	6,630	18,250	4,490	21,700	2,400	8,280	470	4,360
Per cent	6.2	2.7	8.4	1.8	8.8	1.3	4.1	0.3	1.9
Rubriblasts									
Number	1,630	1,000	2,390	200	6,160	500	1,000	0	0
Per cent	0.7	0.4	1.1	0.1	2.5	0.3	0.5	0.0	0.0
Total erythrocyte precursors									
Number	101,350	78,550	112,690	81,510	121,060	51,100	54,330	43,000	83,870
Per cent	43.5	32.0	52.0	32.7	49.1	27.7	26.9	27.6	36.5

When polycythemic serum was used there was a decrease in the number of metarubricytes and rubricytes that was more rapid than that seen in normal cultures during the first 12 hours

DISCUSSION

Normal Serum—The linear decrease of erythrocyte precursors in normal cultures is the product of interaction of several factors The number of precursors present at any time represents the balance between proliferation, cell death, and maturation to anuclear erythrocytes during the preceding period of time Cell death may be large in these cultures because the environmental conditions in the chamber are suboptimal when compared with marrow in situ Proliferation is probably decreased and maturation increased by "progressive maturation of the culture" i e, the tendency of all the cells in the culture to mature and thus become incapable of dividing and maintaining the line Osgood[17] emphasized that when an immature cell divides, one of the daughter cells must remain immature and the other must mature if the normal numerical relationships are to be maintained There is good evidence that this delicate balance is not maintained in these cultures since the proportion of metarubricytes and segmented neutrophils to total erythrocyte precursors and total granulocytes, respectively, increases and the proportion of more immature forms decreases with time Thus all factors tend to decrease the number of erythrocyte precursors during any time interval

Anemic Serum—The increase in the total number of erythrocyte precursors during a 12 hour incubation with anemic serum suggests that the erythropoietic stimulating factors(s) (erythropoietin) is present in anemic serum in a form that is directly usable by the bone marrow cells The proliferative stimulatory effect of this factors(s) is apparently upon the prorubricytes and rubricytes

As in the normal cultures the decline in erythrocyte precursors during the second 12 hours is the result of 3 processes proliferation, cell death, and maturation Again, progressive maturation of the culture is occurring, this tends to offset any stimulatory effect of the serum on proliferation by reducing the number of cells that are capable of division It is impossible to state with certainty whether maturation of single erythrocyte precursors and/or progressive maturation of the culture is hastened by the stimulatory effect of the anemic serum The more rapid decline in rubricytes and prorubricytes during this period suggests that both individual and progressive maturation are probably hastened by the active serum

Cobalt and Cobalt Control Serum—When cobalt serum was used, there was evidence of stimulation to the same degree as in anemic serum When cobalt control serum was used, no such activity was demonstrated These facts substantiate the findings of Goldwasser and associates[9, 18] that the erythropoietic effect of cobalt is mediated through a humoral mechanism (erythropoietin) In addition, the almost exact correspondence of erythrocyte precursors in the cultures grown with cobalt control serum and those grown with normal serum indicates that there is no direct effect of the ion on the bone marrow at these concentrations. These results are in agreement with the observations of Laforet and Thomas[19] who found no direct stimulatory effect of cobalt ion on bone marrow when oxygen levels and heme synthesis were analyzed in cultures.

Polycythemic Serum.—When animals are rendered polycythemic by transfusion, the amount of circulating erythropoietin is presumably reduced because the amount of oxygen at the site of production is increased and thus the stimulus for production is reduced.[11] Erythropoiesis in the transfusion-induced polycythemic mouse spleen is reduced.[10] In the present experiments, the lower number of erythrocyte precursors in polycythemic cultures as compared with that in normal cultures at 12 hours is presumptive evidence that the amount of erythropoietin is greater in normal serum than in polycythemic serum and that erythropoietin is necessary to maintain a "normal" state of erythropoiesis. (However, the presence of an inhibitor of erythropoiesis in the polycythemic serum cannot be ruled out with absolute certainty.) Erythropoietin appears to be one of the controlling mechanisms in the normal state and not merely an emergency mechanism to stimulate erythropoiesis during stress.

Errors of Measurement.—The over-all effects of the various treatments are clear from examination of Figs. 2 to 4. Although 3 chambers were used to determine each total cell count, ordinarily only 1 or 2 differential counts were made for each treatment at each time. Thus, we can measure directly the precision of a total cell count but not that of differential count or of their product, the total erythrocyte precursor count. However, in some preliminary experiments replicate differentials counts were made, and we have used these to measure the precision of differential counts.

It was found that the coefficient of variation (standard deviation divided by the mean count) of repeated total counts was approximately 12 per cent (somewhat smaller for the zero hour counts) and consequently the coefficient of variation of the average of 3 such counts, being less by a factor of $1/\sqrt{3}$, is approximately 7 per cent. For counts in the neighborhood of 200, the standard error is thus approximately 14, and the differences in total count at each time do not greatly exceed what might be expected on the basis of measurement error alone.

The coefficient of variation of repeated differential counts is approximately 14 per cent. Since the total erythrocyte precursor count is the product of the total cell count and the differential count, its coefficient of variation is approximately $\sqrt{7^2 + 14^2}$ per cent, or about 16 per cent. For counts in the neighborhood of 80, the standard error is thus approximately 13, and the differences in erythrocyte precursor total counts are appreciably larger than the estimated magnitude of the measurement error.

SUMMARY

The effects of 5 different types of serum upon erythropoiesis in bone marrow tissue culture have been tested, using a modification of the Mackaness and Smith

chamber Eagle's medium and test serum in a 2 1 proportion were used as nutrient medium Total and differential cell counts were done after 0, 12, 24, and, in one case, 36 hours of incubation at 37° C Cultures sustained with normal rat serum showed a decrease in the number of erythrocyte precursors which is linear and predictable Cultures grown in serum from rats rendered anemic by bleeding showed a marked increase in erythropoiesis after 12 hours of incubation The same effect was seen in cultures grown with serum from rats that had been given cobaltous chloride (25 μm per 100 Gm body wt) 10 hours before the removal of the serum When cobalt ion was added to normal serum in vitro to the same final concentration as found in the serum from injected rats, the resultant combination had no effect on erythropoiesis When serum from rats rendered polycythemic was used, a marked decrease in erythropoiesis was noted after 12 hours of incubation

These experiments give further evidence of the existence of a humoral factor (s) (erythropoietin) that stimulates erythropoiesis, and, in addition, establish that the substance(s) circulates in a form that is readily available to the bone marrow The stimulatory effect of cobalt on erythropoiesis is through this hormone In addition, there is also evidence that erythropoietin is present in serum from normal animals and also that the amount is decreased in serum from animals rendered polycythemic by transfusion

We gratefully acknowledge the efforts of Dr Paul Meier, Department of Statistics of The University of Chicago, in preparing the statistical analyses of these experiments and Dr William Barclay of The University of Chicago School of Medicine for his instruction in the construction and use of the culture chambers

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RESULTS

The redox activity of leukocytes from 256 individuals was determined. Normal values (negative test) were obtained in 222. This group included acute and chronic diseases as well as 84 normal controls. Depressed redox activity (positive test) was found in 34 (Table II).

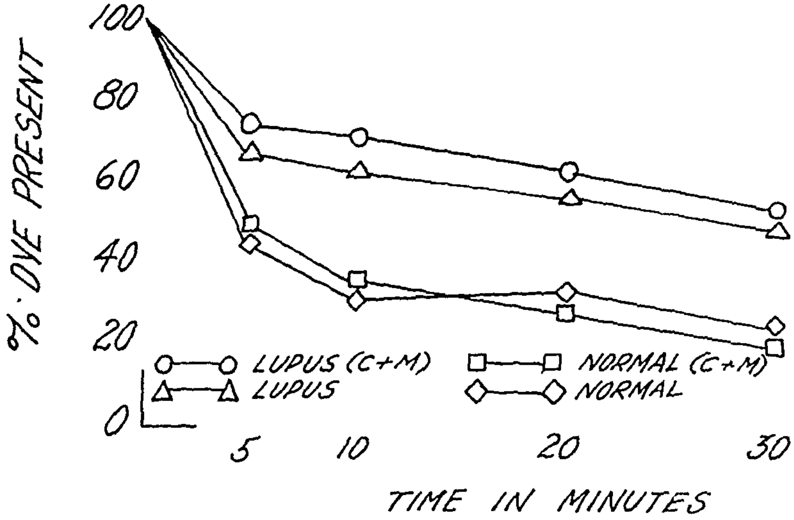

Fig. 2.—Comparisons of "typical" curves from this study (110 normal; 72 "lupus") with those of Chaudhuri and Martin (20 normal; 16 "lupus").

The conditions in which a positive redox test was obtained were: (1) systemic lupus erythematosus; (2) discoid lupus erythematosus; (3) connective tissue disorder, clinically compatible with either systemic lupus erythematosus or severe febrile rheumatoid arthritis, with consistently negative L.E. preparations; and (4) hydralazine syndrome. A total of 57 patients with these disorders were studied and 34 had a positive test.

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CLINICAL DIAGNOSIS	NUMBER OF PATIENTS	REDOX TEST		L.E. CELLS POSITIVE
		POSITIVE	NEGATIVE	
Systemic lupus erythematosus (untreated)	14	14	0	14
Systemic lupus erythematosus (on steroid therapy)	8*	0	8	8
Discoid lupus erythematosus	9	3†	6	0
Differential diagnosis: S.L.E. or severe febrile rheumatoid arthritis	21	12‡	9	0
Hydralazine syndrome	5	5	0	3

*Six additional patients with positive tests prior to treatment had negative tests when receiving steroids.

†All had clinical evidence of dissemination.

‡Eight of these patients have subsequently had positive L.E. cell preparations or positive biopsy.

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DETERMINATION OF REDOX ACTIVITY OF LEUKOCYTES AS A DIAGNOSTIC AID IN SYSTEMIC LUPUS ERYTHEMATOSUS

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IN 1953, Chaudhuri and Martin reported a metabolic alteration in leukocytes from patients with acute disseminated lupus erythematosus, as measured by the redox test.[1] The test is so named because it measures both reduction and oxidation. The leukocytes of patients with systemic lupus erythematosus (S.L.E.) exhibited a depression of activity as compared with the cells obtained from normal subjects. The leukocytes of normal individuals exhibited depression of redox activity following exposure to the plasma of patients with S.L.E.

The present study was undertaken to investigate the specificity of the redox reaction prior to attempts at identification of the plasma factor responsible for the altered metabolism.

MATERIALS AND METHODS

Studies were carried out on healthy medical students, patients with a variety of acute and chronic diseases, patients with systemic lupus erythematosus (untreated and treated with corticosteroids), and patients receiving hydralazine, as shown in Table I. Care was taken to include those entities which fall within the field of connective tissue disorders, those in which an allergic or hypersensitivity mechanism is postulated in regard to etiology, and those in which abnormal serum proteins are commonly found.

Redox activity was measured by the method of Chaudhuri and Martin.[1] Minutiae are added here, as minor technical variations influence the accuracy of the test.

1. Heparin. Liquaemin* sodium (heparin solution), 50 mg. per milliliter.
2. Saline. Sterile physiologic solution (0.9 per cent NaCl).
3. Dextran. A 5 per cent solution is prepared and autoclaved for 20 minutes at 20 pounds pressure (intrinsic viscosity 0.46). This solution will keep for 2 to 3 weeks, if refrigerated.†
4. Dye. *2,6 Dichlorophenol indophenol. For stock solution, 20 mg. are dissolved in 200 ml. of saline.* This solution may be kept for 2 months if refrigerated. For performance of the test, the dye stock solution is diluted 1 6 in saline.‡

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*Organon Inc., Orange, N.J.

†Commercial Solvents Corporation, Terre Haute, Ind.

‡Eastman Organic Chemicals, Rochester 3, N.Y.

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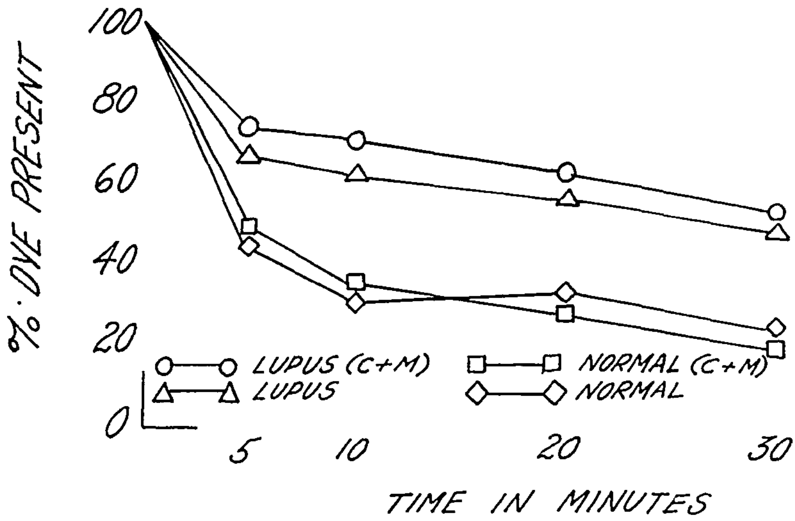

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*Six additional patients with positive tests prior to treatment had negative tests when receiving steroids.

†All had clinical evidence of dissemination.

‡Eight of these patients have subsequently had positive L.E. cell preparations or positive biopsy.

Fourteen patients of this group had systemic lupus erythematosus, L E cells were demonstrable at the time of initial study and all 14 had a positive redox test

In a group of 9 patients with typical skin lesions of discoid lupus erythematosus, 3 positive redox tests were obtained Clinically these 3 individuals had signs of systemic involvement such as leukopenia, albuminuria, fever, and elevated sedimentation rates, but negative L E cell preparations In the remaining 6, there was nothing in the clinical course or laboratory tests to indicate dissemination

Twenty one individuals presented clinically as diagnostic problems with a clinical course compatible with severe febrile rheumatoid arthritis or systemic lupus erythematosus L E cells were not demonstrable at the time of or prior to the performance of the redox test Twelve of these 21 patients had a positive redox test In 8 of these 12, the diagnosis of S L E was substantiated later, not only by the clinical course, but also by the demonstration of L E cells in 6 of the patients, and by biopsies compatible with the diagnosis of S L E in 2 others In one of these patients the positive redox test was obtained 18 months prior to positive pathologic diagnosis At the time the redox test was done, it was difficult to distinguish between the two entities by the clinical pattern of these 21 patients Whereas 8 patients now have definite S L E, it has not as yet been possible to arrive at a more definitive diagnosis in the 13 other "febrile rheumatoids," 4 of whom have a positive and 9 a negative redox test A few case summaries are included to illustrate the difficulty in clinical differentiation

CASE REPORTS

CASE 1 —L E is a 57 year old white saleslady with a 2 month history of fever, bursitis of both shoulders, stiffness and swelling of the proximal interphalangeal joints of both hands, and generalized arthralgias and myalgias, accompanied by a weight loss of 35 pounds Physical examination also revealed pleural effusion in the right chest Laboratory studies revealed the following hematocrit 36, sedimentation rate* 41 mm per hour, white blood count 6,000 with normal differential, STS† negative, total protein 7 Gm per cent with serum albumin 3 8 and globulin 3 3 Gm per cent, gamma globulin 13 units‡, PPD No 1 skin test positive Scalene node biopsy revealed old tuberculoid granulomas L E preparations were negative Redox test was positive

CASE 2 —G P is a 50 year old Negro woman with a 4 month history of fever, swelling, and pain in her ankles, knees, wrists fingers, and elbows, bilateral iritis, and keratitis, accompanied by a weight loss of 20 pounds Laboratory studies were as follows hematocrit 39, sedimentation rate 46 mm per hour, white blood count 7,050 with 5 per cent lymphocytes, STS negative, total protein 7 5 Gm per cent with serum albumin 2 5 and globulin 5 0 Gm per cent, gamma globulin 20 units, sheep cell agglutination positive L E preparations were negative Redox test was positive

Cases 1 and 2 are typical of the "febrile rheumatoids" in whom a positive redox test was found

CASE 3 —G H is a 28 year old Negro stenographer with a 1 year history of pain and swelling of the wrists, fingers, knees, and ankles, following an episode of sore throat,

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*All sedimentation rates (Wintrobe) corrected
†STS = serologic test for syphilis
‡Turbidimetric technique⁴

fever, and left pleural pain. Chest x-ray, taken at time of onset, revealed a left pleural effusion with no evidence of tuberculosis. She had two episodes of parotid gland swelling for which specific etiology could not be determined. Laboratory studies were as follows: hematocrit 34; sedimentation rate 36 mm. per hour; white blood count 3,600 with 65 segmented forms, 5 band forms, and 30 lymphocytes; STS negative; total protein 8.6 Gm. per cent with serum albumin 3.0 and globulin 5.6 Gm. per cent; gamma globulin 21.5 units. L.E. preparations were negative. Redox test was negative.

Case 4.—C. L. is a 20-year-old white female student with a 3-year history of fever, multiple joint swelling, and pain, accompanied by severe fatigue and weight loss. At times, an erythematous skin rash has been noted on the trunk and extremities. Past history revealed that she had acute rheumatic fever at the age of 11. The results of laboratory studies were as follows: hematocrit 34; sedimentation rate 38 mm. per hour; white blood count 8,450 with normal differential; STS negative; total protein 7.3 Gm. per cent with serum albumin 3.4 and globulin 3.9 Gm. per cent; gamma globulin 7 units; antistreptolysin-O titer 166 units; sheep-cell agglutination negative. L.E. preparations were negative, although on one occasion 2 ''tart'' cells were found. Redox test was negative.

Case 5.—W. F. is a 72-year-old white man with an 18-month history of fever, joint pain, and swelling involving the knees, ankles, and proximal interphalangeal joints of the fingers, elbows, and shoulders, accompanied by a 40-pound weight loss. Laboratory studies revealed: hematocrit 36; sedimentation rate 44 mm. per hour; white blood count 10,500 with a slight shift to the left on differential; total protein 6.5 Gm. per cent with serum albumin 3.0 and globulin 3.5 Gm. per cent; gamma globulin 21 units; STS negative; sheep-cell agglutination positive. Chest x-rays have revealed a fibrotic infiltration in both lung fields which has gradually increased over a period of 12 months. Repeated studies for tuberculosis have been negative. L.E. preparations were negative. Redox test was negative.

These 3 cases are typical of the "febrile rheumatoids" in whom a negative redox test was found. The similarity of the clinical pattern with Cases 1 and 2 can be seen, the only significant difference being the results of the redox test. To date, L.E. cell preparations have remained negative in all 5 and there have been no differentiating features in the clinical course.

Case 6.—L. F. is a 66-year-old white woman with a 1-year history of pain, stiffness, and swelling of the hands, shoulders, knees, and ankles, as well as stiffness of the neck, accompanied by fever, weight loss, and repeated episodes of nonspecific abdominal pain. Laboratory studies: hematocrit 28; sedimentation rate 36 mm. per hour; white blood count 5,200 with 76 segmented forms, 3 band forms and 21 lymphocytes; total protein 6.7 Gm. per cent with serum albumin 2.5; and globulin 4.2 Gm. per cent; gamma globulin 7.5 units; STS negative; sheep-cell agglutination negative. L.E. preparations were negative. Redox test was positive. Her course was progressively downhill. She developed marked anorexia and irritability. She was hospitalized 6 months later and restudied. X-rays showed bilateral lung involvement compatible with virus pneumonia or malignancy. Fluid was noted in both pleural cavities. All studies for tuberculosis were negative. L.E. preparations were now positive. Redox test remained positive. Therapy with corticosteroids was instituted. Redox test was repeated after steroids were administered and was negative. L.E. cell preparations were repeatedly positive, while on steroid therapy.

This case serves to illustrate the demonstration of a positive redox test in a "febrile rheumatoid" approximately 7 months prior to the demonstrations of positive L.E. cell preparations. In another instance, a positive redox test has been obtained 18 months prior to definitive diagnosis of S.L.E.

Six patients with S.L.E., postive L.E. preparations, and positive redox tests were examined again after they had been placed on corticosteroid therapy In each case the positive curve reverted to normal after steroid administration (Fig 3)

In one patient the redox test was found to be negative 4 hours after a single intravenous dose of 50 mg of hydrocortisone As little as 5 mg of prednisone per 24 hours was found to cause reversion of the abnormal redox activity to normal Eight additional patients with S L E and demonstrable L E cells were seen by us only after hormonal therapy had been started All 8 patients had a negative redox test

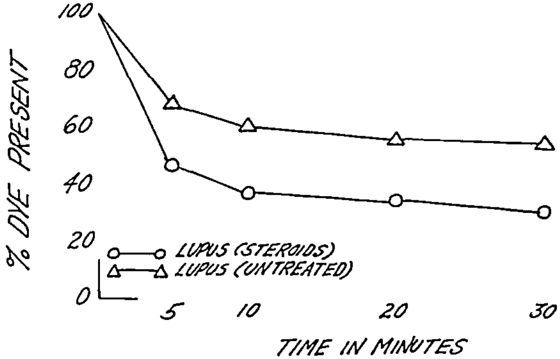

Fig 3.—Redox curves of 6 patients with S L E (18 determinations for each curve) before and after treatment with steroids (See text)

Five of 11 patients receiving hydralazine had a positive redox test Four of the 5 had symptoms of the hydralazine syndrome at the time of study, L E cells were demonstrated in 3 The fifth individual was selected because he was receiving hydralazine in doses of 800 mg daily He was found to have positive L E preparations and a positive redox test, despite the absence of symptoms of the hydralazine syndrome The 6 patients with a negative redox had negative L E cell preparations and no symptoms

The dosage range of hydralazine was in excess of 300 mg per day in 4 of the 5 patients The other patient had had a severe hydralazine reaction 2 years prior to this study, following prolonged administration of the drug at levels of 600 mg daily No hydralazine was given in the 2 year interval Following determination of redox activity, which was normal, a single 50 mg dose of hydralazine was administered The patient developed symptoms of the hydralazine syndrome and a positive redox test within 24 hours L E cells were not demonstrable

Depressed redox activity, symptoms of hydralazine syndrome and positive L E preparations, when present, reverted to normal in all instances when hydralazine was withdrawn (Fig 4)

DISCUSSION

In recent years, a number of laboratory tests have been introduced in the field of connective tissue disease. The attempt is usually made to use these tests for a dual purpose, namely, as an aid in diagnosis and as a tool in the study of pathophysiologic processes. The data presented here indicate that the redox test may be a useful adjunct in the diagnosis and study of these diseases. Since the description of the L.E. cell by Hargraves and associates[5] many clinical syndromes have been identified as S.L.E., using the L.E. cell test as the principal criterion for diagnosis.

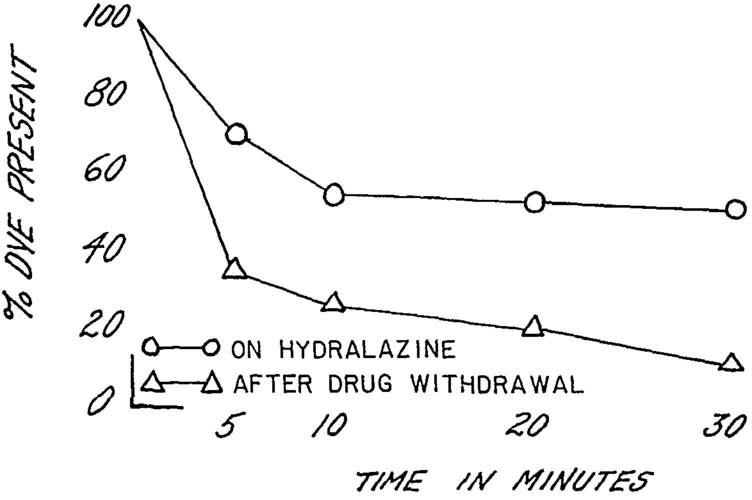

Fig. 4.—Redox curve of 3 patients with hydralazine syndrome (17 determinations for each curve) before and after (2 to 11 days) hydralazine withdrawal.

In comparison to the L.E. cell test, the redox test is technically more difficult to perform. Its use, therefore, solely as a diagnostic procedure in patients with an established clinical diagnosis of S.L.E. and positive L.E. cell preparation does not seem warranted. However, it may be of diagnostic value in those patients who have a clinical picture suggestive of S.L.E. but with negative L.E. preparations. Further, it may aid in the differentiation of those patients with diffuse systemic rheumatoid disease with positive L.E. cell preparations. The redox test has an advantage in accuracy, in that the test results are determined spectrophotometrically and are, therefore, not subject to the variations of technique and interpretations of suspicious or abnormal cell morphology. As stated by the editors of the Eleventh Rheumatism Review, "The status of the L.E. cell in rheumatoid arthritis is confusing because of the varied methods employed in performing the test, and because there is not complete agreement as to the characteristics which identify the L.E. cell."[6] Much the same comment might be made in regard to specificity. Positive L.E. cell preparations have been reported in penicillin sensitivity,[7] generalized moniliasis,[8] hydralazine syndrome,[9-11] diffuse systemic rheumatoid disease,[12] multiple myeloma,[13] pernicious anemia,[14] discoid lupus erythematosus,[14] fungus contamination,[15] Senear-Usher pemphigus,[16] miliary tuberculosis,[17, 18] dermatitis herpetiformis,[14] leukemia,[19] and reaction to hydantoin,[20] as well as S.L.E. To the present time, the redox test has been found positive only in systemic lupus erythematosus (nonsteroid treated), in discoid lupus when there was evidence of dissemination, in patients with unclassified "febrile rheumatoid arthritis" whose clinical course was compatible with S.L.E., and in patients receiving hydralazine when symptoms of hydralazine reaction have developed. It would appear that a positive redox test is highly suggestive of S.L.E. Thus far, we have not encountered a patient who had untreated S.L.E., positive L.E. cell preparations, and a negative redox test.

The group of patients with unclassified "febrile rheumatoid arthritis" has been of particular interest. Application of the redox test has allowed division into two subgroups, those with a positive and those with a negative test. With respect to the clinical picture there were no differentiating features. In 8 of 12 patients who had a positive test, the diagnosis of S.L.E. was established at a later date, whereas all patients with a negative test have remained unclassified, both with regard to laboratory studies and clinical course.

As noted above, the redox test has also been found positive in patients receiving hydralazine, when symptoms of hydralazine reaction have developed. The significance of hydralazine reaction is not clearly defined. It has generally been considered that the reaction appears only after prolonged drug administration and, therefore, differs in mechanism from the usual type of hypersensitivity reaction to drugs.[21-23] In our experience there was no consistent correlation of positive redox test to dosage or duration of therapy. Thus far, the positive redox test correlated best with the development of symptoms of hydralazine syndrome. The one patient with a positive test who had no symptoms had positive L.E. preparations.

In further studies we have confirmed the observation that plasma from patients with systemic lupus erythematosus induces altered redox activity of normal leukocytes.[1] Pilot studies indicate that this same alteration is induced in normal leukocytes by plasma from patients with the hydralazine syndrome. The altered redox activity of leukocytes, therefore, appears to be attributable to a plasma factor rather than to an intrinsic defect of the white cell. It is of interest that a similar plasma factor can be postulated in both a drug-induced state and a naturally occurring disease. Further study will be necessary to learn more about this factor in both conditions.

Redox activity of leukocytes returns to normal when corticosteroids (cortisone, ACTH, hydrocortisone, prednisone, and prednisolone) are given to patients with S.L.E. or when hydralazine is withdrawn in patients with the hydralazine syndrome. Reversal of drug-induced abnormal redox activity to normal by factors other than drug withdrawal is now under study.

The change in redox activity of leukocytes reflects a profound alteration in cell metabolic structure.[1] At the present time the site and the reason for this alteration are not certain. As it is possible to induce this alteration in normal leukocytes with the plasma of patients with S.L.E. and hydralazine syndrome, it will be of interest to attempt to isolate, identify, and further study this plasma factor and its effect on cells.

SUMMARY

The redox activity of leukocytes from 256 individuals has been studied. Depressed redox activity was found in 34. Abnormal activity was found in all of 14 cases of nonsteroid-treated typical systemic lupus erythematosus studied and in 8 additional patients with S.L.E. prior to definite diagnosis by currently acceptable methods. When positive redox tests were obtained in discoid lupus erythematosus, there were other data suggesting systemic involvement. In 4 of 13 "febrile rheumatoids," a positive redox test was demonstrated. Thus far no other differentiating features in the clinical course or laboratory findings have developed in this group of patients. Five patients receiving hydralazine demonstrated altered redox activity as well as symptoms of hydralazine reaction and/or positive L.E. preparations.

It is suggested that depression of redox activity of leukocytes, a positive redox test, may be specific for systemic lupus erythematosus and hydralazine reaction, and could, therefore, serve as a useful laboratory adjunct in early diagnosis.

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A MICROMETHOD OF EUGLOBULIN FIBRINOLYSIS IN PLASMA OF HUMAN SUBJECTS AND SMALL LABORATORY ANIMALS

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There exists a need for a micromethod to determine fibrinolytic activity. In an attempt to develop such a method, the macromethod described by Kowarzyk and Buluk[1] and particularly used by one of us[2-4] appeared to be suitable for adaptation to microvolumes. In this test the euglobulin fraction is precipitated by acetic acid and coagulated by calcium chloride. Fibrinolysis times of these euglobulin coagulates are then determined. This communication presents comparisons of plasma euglobulin fibrinolysis in human subjects, rabbits, guinea pigs, rats, and hamsters, using macro- and microvolumes of plasma

METHOD

Fresh oxalated plasma (1 volume part 0.1 M ammonium oxalate and 9 volume parts blood) is obtained by centrifugation for 10 minutes at 1,500 r.p.m. In human subjects blood was drawn from the antecubital vein or the end phalanx of the finger, in which a large enough wound was inflicted to secure a free flow of blood. The blood was drawn from the ear vein and the heart of rabbits and from the heart of guinea pigs, rats, and hamsters

The two different procedures used are described in the following sections

A Euglobulin Fibrinolysis in Human, Rabbit, Rat, and Hamster Plasma.—Tests in duplicate or triplicate are carried out as follows. To 0.05 ml. plasma in Pyrex tubes measuring 8 by 100 mm, add 0.9 ml. distilled water, containing 0.015 ml. of 1 per cent acetic acid. (The acidified water is prepared beforehand by mixing 90 ml distilled H_2O with 1 ml. 1 per cent CH_3COOH.) The pH, which should be determined, is approximately 5.2. After these tubes have been kept for 30 minutes in a refrigerator at +4° C., they are centrifuged for 5 minutes at 1,500 r.p.m. The tubes are emptied of the entire supernatant, then placed upside down on filter paper to drain for about one minute, during which time the sediment adheres strongly to the wall of the tube. With the aid of a fine glass rod, the sediment is dissolved in 0.05 ml borate solution of pH 9.0, which is composed of 9 Gm sodium chloride and 1 Gm. sodium borate in 1,000 ml distilled water The tubes are then placed in a water bath at 37° C and, 3 to 5 minutes later, 0.05 ml. of 0.025 M calcium chloride is added to the tubes which are maintained at a constant temperature. A coagulate forms within a few minutes With all other plasma volumes employed, the volumes of the different solutions have to be adjusted accordingly. The tubes are kept in the water bath and observed every 5 minutes in order to see when the coagulate dissolves

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B Euglobulin Fibrinolysis in Guinea Pig Plasma or Serum.—To 0.05 ml of plasma or serum, 1.0 ml of acidified water is added. The pH of the mixture is 5.4. After the tubes *have been kept for 18 hours in a refrigerator at 4° C, they are centrifuged.* The sediment is then dissolved in 0.2 ml borate solution and fibrinolysis is examined as soon as possible in a system composed of 0.1 ml euglobulin, 0.2 ml borate solution, 0.1 ml thrombin (10 units per milliliter), 0.4 ml of 0.2 per cent fibrinogen. The bovine fibrinogen employed is prepared by the method of Kekwick and associates[5] and contains 96 per cent clottable nitrogen. With all other plasma volumes employed, the volumes of the different solutions have to be adjusted accordingly. The tubes are kept in a water bath at 37° C and observed every 15 seconds, the lysis of the coagulate being noted.

RESULTS

Table I shows duplicate results of euglobulin fibrinolysis employing from 0.5 to 0.02 ml of human plasma from 4 healthy subjects. As can be seen, duplicate tests usually give rather close values of fibrinolysis time. Volumes of 0.02 ml cannot be recommended since, as is also shown in Table I, in some instances the euglobulin solution does not coagulate. In all our tests euglobulin fibrinolysis times were found from 130 to 240 minutes.

TABLE I EUGLOBULIN FIBRINOLYSIS IN DUPLICATE TESTS EMPLOYING VARYING AMOUNTS OF HUMAN PLASMA

PLASMA (ML.)	TUBE	FIBRINOLYSIS TIME (MINUTES) IN HUMAN SUBJECTS			
		A	B	C	D
0.5	1	175	185	185	185
	2	185	175	185	195
0.3	1	170	190	200	185
	2	180	195	200	190
0.2	1	165	190	200	170
	2	175	185	200	185
0.1	1	180	200	240	185
	2	170	190	225	170
0.05	1	170	190	235	150
	2	175	185	200	150
0.02	1	—*	135	130	—*
	2	—*	145	135	—*

*Euglobulin solution did not coagulate.

TABLE II COMPARISON OF EUGLOBULIN FIBRINOLYSIS IN PLASMA SECURED FROM HUMAN VENOUS AND CAPILLARY BLOOD

BLOOD	PLASMA (ML.)	FIBRINOLYSIS TIME (MINUTES) IN HUMAN SUBJECTS					
		I	II	III	IV	V	VI
Venous	0.5	220	245	225	205	230	160
	0.05	245	195	240	210	225	180
Capillary	0.05	220	190	245	205	230	150

In Table II a comparison is given of fibrinolysis values in the plasma secured from the venous and capillary blood of 6 normal human subjects. As can be seen, there is no significant difference in fibrinolysis times between so called *"capillary" blood secured from the finger tip and venous blood.*

In the rabbit both macro- and microvolumes were found to be applicable only if the following procedure is carried out. The centrifuged sediment is incubated in borate solution for about 2 hours in the refrigerator; otherwise, it does not dissolve readily in the borate solution. Using this modification, the lysis time employing varying volumes of plasma (shown in Table III) was found to be not as constant as with human or hamster plasma.

TABLE III. EUGLOBULIN FIBRINOLYSIS TIME IN TESTS EMPLOYING VARYING VOLUMES OF RABBIT PLASMA

PLASMA (ML.)	FIBRINOLYSIS TIME (HOURS) IN RABBITS						
	I	II	III	IV	V	VI	VII
0.5	4.4	5.0	4.2	4.6	6.3 IC	3.8	6.3
0.3	5.9	6.2	6.5 IC	3.3	5.8	6.1	4.7
0.2	3.8	4.5	6.5 IC	6.5 IC	3.8	6.3 IC	3.2
0.1	5.9	3.9	4.0	3.3	3.1	3.8	4.8
0.05	4.3	4.5	3.2	4.5	3.1	5.1	5.1
0.02	3.3	3.2	3.2	4.2	1.1	3.1	4.0

IC = Incomplete lysis; in all these instances, complete lysis was found after 21 hours.

In Table IV plasma euglobulin fibrinolysis employing 0.05 ml. plasma secured from blood drawn on different days from the hamster's heart shows that there is no significant difference in values. Not shown in Table IV are tests using varying volumes of 0.3, 0.1, 0.05, 0.03, and 0.01 ml. With the exception of the latter volume, all others exhibited similar fibrinolysis times. In the tests employing 0.01 ml. plasma, the euglobulin solution did not coagulate. Euglobulin fibrinolysis times varied from 75 to 140 minutes.

TABLE IV. EUGLOBULIN FIBRINOLYSIS MICROTESTS EMPLOYING PLASMA SECURED FROM BLOOD DRAWN ON DIFFERENT DAYS FROM THE HAMSTER'S HEART

HAMSTER	FIBRINOLYSIS TIME IN MINUTES	
	AUGUST 24	AUGUST 26
I	115	105
II	115	120
III	125	115
IV	110	125

Our findings with guinea pig plasma showed that the euglobulin solution *does not coagulate.* Guinea pig euglobulins show potent fibrinolytic activity, which suggests that their own fibrinogen is readily digested. This activity may be determined after the addition of thrombin and fibrinogen preparations to the euglobulin solution.

Table V shows the results of serum euglobulin fibrinolysis of 4 healthy and fasting guinea pigs. Volumes from 0.5 to 0.05 ml. guinea pig serum were employed. When guinea pig plasma is used instead, no significant difference in values has been obtained. For this animal, therefore, serum, which is more convenient for practical reasons, can be used instead of plasma.

The euglobulin fibrinolysis method cannot be recommended for testing rat plasma. The coagulates formed from rat plasma euglobulins are very resistant to fibrinolysis, since lysis times varied from 12 to 48 hours and more.

Since we found the use of 0.05 ml plasma adequate in order to obtain results similar to those acquired with 0.5 ml we adopted the 0.05 ml plasma sample for the micromethod. If volumes of 0.02 ml or even 0.01 ml were used, the lysis times, wherever the euglobulin solutions could be coagulated, differed often from those obtained with the 0.05 ml volume.

TABLE V EUGLOBULIN FIBRINOLYSIS TESTS EMPLOYING VARYING VOLUMES OF GUINEA PIG SERUM

SERUM (ML.)	FIBRINOLYSIS TIME (SECONDS) IN GUINEA PIGS			
	I	II	III	IV
0.5	390	450	615	510
0.2	390	—	690	510
0.1	420	480	750	—
0.05	480	570	615	570

DISCUSSION

Kopec and Niewiarowski,[4] using the macromethod, found the normal fibrinolysis time for human subjects to be 219 minutes ± 5 minutes with a standard deviation ± 61 minutes. According to these authors, the fibrinolysis time in the euglobulin plasma fraction is influenced by many factors, such as the concentrations of fibrinolytic enzymes and fibrinogen, the pH with an optimum of 7.6, temperature, and the time interval elapsing between the collection of blood and the preparation of euglobulins. In normal subjects, this time interval does not appear to be significant. However, if fibrinolysis is activated, the blood samples *should be preserved in an ice water bath or the determination should be* made immediately. Therefore, all tests were run directly after the blood was secured.

From observations by Milstone[6] and by Macfarlane and Biggs it is well known that plasminogen is a chief component of the euglobulin fraction. However, the euglobulins are not completely free from antiplasmin and M. Niewiarowska found that the antiplasmin level influences the fibrinolysis time.[3] Also the activation of fibrinolytic enzymes in euglobulins is influenced by coagulation.[1, 2]

Although fibrinolysis in euglobulin and whole plasma does not always run parallel, as shown for instance by Ratnoff,[7a, 7b] fibrinolysis of the euglobulin fraction appears to be affected by factors similar to those in fibrinolysis of whole plasma in accordance with findings by Kopec and Niewiarowski[4] and Niewiarowska.[8] However, as experienced by these authors, the euglobulin method appears to be more sensitive and to give more consistent results than methods employing whole plasma. The euglobulin method can also be extended to the study of fibrinolysis in other animals.

Subsequent to these observations the reason for the failure of formation of a coagulate of guinea pig euglobulin has been found in studies of the fibrinolytic system in the guinea pig by Latallo, Niewiarowski, and Copley.[9] It was found that guinea pig plasma contains a plasmin antiplasmin complex which dissociates readily after dilution at pH 5.3 and that the euglobulin fraction

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0.02	3.3	3.2	3.2	4.2	1.1	3.1	4.0

IC = Incomplete lysis; in all these instances, complete lysis was found after 21 hours.

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THE PHOTOMETRIC DETERMINATION OF THE SIALIC (N-ACETYLNEURAMINIC) ACID DISTRIBUTION IN CEREBROSPINAL FLUID

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NEURAMINIC acid, a complex amino acid sugar (its acetylated derivatives designated as sialic acid), is a functional group of many glycoproteins and glycolipids found in a variety of biologic fluids and tissues.[1-3] Its importance in neurologic problems is shown by the fact that it constitutes an integral part of the "gangliosides" which are complex lipids found in brain tissue.[4, 5] These gangliosides have been found to be increased in certain neurologic conditions, including Niemann-Pick disease and amaurotic familial idiocy (A.F.I.). In some instances they completely displace the normal cerebrosides and phospholipids of brain gray matter.[6, 7]

A number of investigators[8-11] have shown that N-acetylneuraminic acid is present in blood serum in such tightly bound combination with proteins that it is both nondialyzable and completely precipitable with trichloroacetic acid. The presence of sialic acid in cerebrospinal fluid (C.S.F.) has been demonstrated by Roboz and associates[12, 13] and by Uzman and Rumley.[14] However, the latter investigators showed that in the C.S.F. 60 to 80 per cent was present in a form which was dialyzable and not precipitable with trichloroacetic acid. Both groups of investigators employed methods which required large amounts (2 to 6 ml.) of C.S.F. for each analysis and use the relatively insensitive and nonspecific orcinol reaction for the colorimetric determination of its sialic acid content. The results reported by these experimenters[12-14] for individual cases held forth the promise that the C.S.F. might be expected to reflect certain pathologic disorders of the central nervous system to a greater extent than would the serum.[15] It was in order to test this hypothesis that the sensitive and specific diphenylamine reaction,[16] previously employed for serum analysis in our laboratory,[17, 18] was adapted to the quantitative determination of total sialic acid (TSA), protein-bound sialic acid, and the nonprotein-bound sialic acid, by difference, in cerebrospinal fluid. The method, as finally evolved, requires only 1.5 ml. of C.S.F. for the sialic acid determinations as well as for the total protein (TP) content.

The present paper deals with the results obtained with these procedures for the C.S.F. from a large number of patients with various neurologic diseases as well as for a control group of nonneurologic cases. In a number of

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instances, sialic acid values were determined for both serum and C S F drawn simultaneously from the same individual Evidence is presented for the possible diagnostic usefulness of such determinations in various neurologic diseases with special reference to amaurotic familial idiocy and related disorders

METHODS

A *Reagents and Apparatus*—

1 Sodium chloride 0 85 per cent solution

2 Sodium hydroxide 10 N solution

3 Biuret reagent (modified after Gornall and associates[19]) In a liter volumetric flask containing about 500 ml of distilled water, place 3 0 Gm of $CuSO_4 \cdot 7\ H_2O$ and 12 Gm of sodium potassium tartrate (Rochelle salt), shake until dissolved Then, with shaking add 300 ml of 2 5 N NaOH Add 2 Gm KI, shake until dissolved, and dilute to volume with distilled water

4 Total protein standard Reconstitute a prepared serum standard, e g, Versatol,* with 5 0 ml of a solution composed of 10 per cent NaCl plus 1 0 per cent of disodium ethylenediaminetetraacetic acid Refer to manufacturer's chart for protein content Dilute 1 0 ml to 100 ml with 0 85 per cent NaCl in a volumetric flask Keep refrigerated when not in use and discard when cloudy

5 Trichloroacetic acid (TCA) 5 0, 7 5, and 10 per cent solutions were prepared by dilution from a 90 per cent stock solution

6 Diphenylamine (DPA) reagent (1 0 per cent) Recrystallize diphenylamine twice from hot ethanol to remove impurities Dissolve 1 0 Gm in a mixture of 90 ml of glacial acetic acid plus 10 ml of concentrated sulfuric acid

7 Sialic (*N* acetylneuraminic) acid standard A purified orosomucoid protein sample was prepared from outdated blood bank plasma by the procedure by Weimer and associates[20] This preparation has a reported sialic acid content of about 11 2 per cent and was checked against a reference sample supplied by Dr R J Winzler For a 10 mg per cent standard dissolve 8 9 mg of the orosomucoid preparation in 10 ml of distilled water Five and 2 5 mg per cent standards were then prepared by dilution from this solution The 10 mg per cent standard solution will remain stable for about one week if kept frozen between runs

8 Absolute ethyl alcohol

9 Evaporation caps to fit 13 by 125 mm Pyrex tubes†

B *Determination of Total Protein Content of Cerebrospinal Fluid*—Pipette 1 0 ml of C S F samples into small test tubes calibrated at 2 0 ml Add 1 0 ml of 10 per cent TCA to each tube, and let stand at room temperature for 15 minutes Centrifuge precipitates at 3,000 r p m for 10 minutes Decant off supernatant fluids and dissolve precipitates in one drop of 10 N NaOH by shaking tubes until precipitates are dissolved Add 1 0 ml of biuret reagent, and dilute to 2 0 ml mark with 0 85 per cent NaCl solution Mix well, let stand for 20 minutes, and read in a spectrophotometer at 530 mμ, or in a Klett Summerson colorimeter, in a flat bottom colorimeter tube, with a No 54 filter against a saline blank (1 0 ml 0 85 per cent NaCl plus 1 0 ml biuret reagent) which has been set at zero The TP standards are run simultaneously and in exactly the same manner as were the C S F samples

Calculations Total protein (C S F) mg per cent $= \frac{\text{O D unknown}}{\text{O D standard}} \times$ total protein standard (mg per cent)

C *Determination of Total Sialic Acid (TSA) Content of Cerebrospinal Fluid*—Pipette 0 5 ml of C S F samples into 15 ml centrifuge tubes Add 1 0 ml of 7 5 per cent TCA to each tube Mix and cover tubes with glass evaporation caps Heat tubes in a boiling

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*Versatol is sold by Warner Chilcott Laboratories Morris Plains N J

Obtainable from Kopp Laboratory Supplies 70 13 35th Rd Queens 72 N Y water bath for 15 minutes. Cool tubes in cold tap water and centrifuge precipitates* at 3,000 r.p.m. for 10 minutes. Pipette 1.0 ml. aliquots of the supernatant fluid into 13 by 125 mm. test tubes. Add 2.0 ml. of the DPA reagent and mix. Cover each tube with an evaporation cap and place in a boiling water bath for 30 minutes. Cool to room temperature in a cold water bath. A reagent blank (0.5 ml. water) and 0.5 ml. aliquots of 5 mg. per cent and 10 mg. per cent SA standards, in duplicate, are carried through exactly the same procedure as are the C.S.F. samples. The optical density values of the unknowns and standards are then read in a spectrophotometer at 530 mμ (or in a Klett photoelectric colorimeter with a No. 54 filter) against a reagent blank set at zero.

Calculations: Total sialic acid (C.S.F.) mg. per cent $= \frac{\text{O.D. unknown}}{\text{O.D. standard}} \times 10.0$ (or 5.0). Since the color reaction deviates from Beer's law, the standard used in making the calculations should be that which is closest to the unknown.

D. *Determination of Protein-Bound Sialic (N-Acetylneuraminic) Acid Content of Cerebrospinal Fluid.*—Into 15 ml. centrifuge tubes pipette 1.0 ml. aliquots of the C.S.F. and add to each tube 4.0 ml. of absolute alcohol. Mix contents and place tubes in a 65° C. water bath for 20 minutes. Cool tubes to room temperature in cold tap water and centrifuge at 3,000 r.p.m. for 10 minutes. Decant off supernatant fluid as completely as possible and break up precipitates by tapping bottom of the tube. Dry the precipitates by placing tubes in a boiling water bath until no alcohol odor can be detected. Add 1.5 ml. of 5.0 per cent TCA to each tube containing the alcohol precipitates. Pipette, in duplicate, into 15 ml. centrifuge tubes 0.5 ml. of distilled water (for reagent blank) and 0.5 ml. of 2.5 and 5.0 mg. per cent SA standards. Add 1.0 ml. of 7.5 per cent TCA to blank and standard tubes *only.* Mix and cover mouths of all tubes with evaporation caps. Heat all tubes in a boiling water bath for exactly 15 minutes. Cool tubes to room temperature in cold tap water and centrifuge at 3,000 r.p.m. for 10 minutes. Pipette 1.0 ml. aliquots of the supernatant fluid into 13 by 125 mm. test tubes. Add 2.0 ml. of DPA reagent and mix. The remainder of the procedure is exactly as described for the TNA determination in the preceding paragraph (C).

Calculations: Protein-bound sialic acid (mg. per cent) $= \frac{\text{O.D. unknown}}{\text{O.D. standard}} \times 2.5$ (or 1.25).

E. *Total Protein and Total Protein-Bound, and Nonprotein-Bound Sialic Acid Content of the Cerebrospinal Fluid of Patients With Various Neurologic Diseases.*—C.S.F. was obtained by lumbar puncture from patients with a wide variety of neurologic and nonneurologic diseases. Bloody fluids and those with high cell counts were eliminated from these studies and all fluids were centrifuged prior to analysis. The total protein and total, protein-bound, and nonprotein-bound sialic acid values were then determined for each individual sample, usually in duplicate, by means of the procedures described above. In a number of instances serum total sialic acid and globulin sialic acid values were obtained by previously published procedures[17, 18] on blood samples drawn simultaneously with the C.S.F.

F. *Determination of "Nondialyzable" Protein-Bound Sialic (N-Acetylneuraminic) Acid Content of Cerebrospinal Fluid.*—Six pooled samples of C.S.F. were analyzed, in duplicate, for their protein-bound sialic acid content with the procedure described before in Paragraph D. Five milliliter aliquots of each of the 6 pooled samples were placed in cellophane bags and dialyzed for 16 hours in cold, running tap water in order to remove any nonprotein-bound sialic acid from the fluids. The contents of the bags were transferred to 5.0 ml. calibrated tubes and, after the addition of concentrated salt solution so as to give a final concentration of 0.9 per cent NaCl, adjusted to their original volume with water. This "nondialyzable" protein-containing C.S.F. fraction was then analyzed, in duplicate, for its sialic acid with the procedure described in Paragraph C, except that 1 ml aliquots were used The results obtained in these runs are given in Table I togeth with the original total sialic acid values of each pooled sample

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*To conserve C.S.F., the dried precipitates obtained in this step can be dissolved in one drop of 10 N NaOH with shaking; the remainder of the procedure is carried out as described above for the T.P. determination except that the results are multiplied by 2.

TABLE I COMPARISON OF PROTEIN BOUND SIALIC ACID VALUES OBTAINED WITH ALCOH PRECIPITABLE FRACTION (AT 65°) AND WITH THE "NONDIALYZABLE" FRACTIONS OF THE SAME CEREBROSPINAL FLUID SAMPLES

SAMPLE NO	TOTAL SIALIC ACID (MG %)	PROTEIN BOUND SIALIC ACID (ALCOHOL PRECIPITABLE FRACTION) (MG %)	PROTEIN BOUND SIALI ACID ("NONDIAYZABLE" FRACTION) (MG %)
1	6 9	1 2	1 4
2	8 9	1 8	1 8
3	6 8	0 9	1 1
4	5 7	0 9	1 0
5	10 5	1 6	1 6
6	6 2	1 1	1 2

RESULTS

For the patient control group "presumed" normal cerebrospinal flui were selected mainly from among nonneurologic cases having TP values b tween 20 to 45 mg per cent The mean value of 29 0 ± 1 6 obtained for th control group (Table II) is considerably lower than the value of 37 5 ± 1 previously reported from this laboratory[15] However, the TP values were o tained by application of the biuret color reaction to the proteins *precipitat* by 5 per cent trichloroacetic acid Some biuret reacting glycoproteins ar polypeptides are not precipitated in strongly acidic solutions[1, 14] and in pa the decreased values may be due to the elimination of the faint turbidi which occurs upon the addition of many biuret reagents directly to the C S F Consequently the mean C S F total protein value for the amaurotic famil idiocy group, i e , 37 3 ± 3 2 mg per cent with this modified procedure is al lower than that reported in our previous paper[1] i e 41 7 ± 2 5 mg per cen

As can be seen from the statistical data in Table II both Group A (vasc lar diseases of the central nervous system) and Group B (demyelinating di eases) have somewhat elevated TP values whereas Group D (cases wit extensive brain damage) exhibits markedly elevated C S F total prote values These groups were included in this study in order to permit an i vestigation of the C S F sialic acid distribution over a wide range of T values and the inclusion of many different types of neurologic disorders I agreement with the reported results of Uzman and Rumley[14] in 49 sampl of human C S F , those obtained in this study for the 46 cases in Groups B, C, and E show that 60 to 80 per cent is present in a form which is n bound by the C S F proteins Group D, the high TP group, shows only 4 to 70 per cent of the total sialic acid present to be in an unbound state

The mean C S F total sialic acid for the control group, i e , 5 1 ± 0 3 m per cent obtained with the diphenylamine method is markedly increased ov the range of 1 1 to 3 4 mg per cent reported by Uzman and Rumley[14] wit the orcinol procedure of Bohm and associates[27] A similar difference of valu for serum total sialic acid has also been found between the two procedur

TABLE II. GROUP STATISTICAL DATA OF CEREBROSPINAL FLUID PROTEINS AND SIALIC ACID VALUES IN 42 PATIENTS WITH VARIOUS NEUROLOGIC DISEASES AS COMPARED WITH A CONTROL GROUP OF 15 "PRESUMED" NORMAL SUBJECTS

DISEASE GROUPS	TOTAL PROTEIN (MG. PER CENT)	TOTAL SIALIC ACID (MG. PER CENT)	PROTEIN-BOUND SIALIC ACID (MG. PER CENT)	NONPROTEIN-BOUND SIALIC ACID (MG. PER CENT)	TOTAL SIALIC ACID / TOTAL PROTEIN (PER CENT)	PROTEIN-BOUND SIALIC ACID / TOTAL SIALIC ACID (PER CENT)
A. Vascular diseases of central nervous system (parkinsonism, cerebrovascular disorders, etc.) 11 cases	34.7 ± 3.8* 24.1 – 45.3† N.S.‡	6.7 ± 0.4 5.6 – 7.8 <0.01	1.4 ± 0.08 1.2 – 1.6 N.S.	5.3 ± 0.4 4.2 – 6.4 N.S.	20.9 ± 1.8 15.9 – 25.9 N.S.	20.9 ± 1.2 17.5 – 24.3 N.S.
B. Demyelinating diseases (multiple sclerosis, Schilder's disease, etc.) 9 cases	34.8 ± 3.9* 33.7 – 35.9† N.S.‡	6.4 ± 0.8 4.1 – 8.7 N.S.	1.1 ± 0.1 0.8 – 1.4 N.S.	5.6 ± 0.7 3.6 – 7.6 N.S.	19.6 ± 2.0 13.9 – 25.3 N.S.	16.7 ± 1.3 13.0 – 20.4 N.S.
C. Amaurotic familial idiocy (Tay-Sachs disease) 12 cases	37.3 ± 3.2* 28.3 – 46.3† N.S.‡	3.7 ± 0.4 2.6 – 4.8 <0.01	0.8 ± 0.06 0.6 – 1.0 <0.01	3.0 ± 0.3 2.2 – 3.8 <0.01	10.2 ± 0.7 8.2 – 12.2 <0.01	21.4 ± 1.7 16.6 – 26.2 N.S.
D. Cerebral degeneration—extensive (hydrocephalus, Niemann-Pick, craniopharyngioma, etc.) 10 cases	151 ± 22.7* 87.2 – 214.8† <0.01‡	8.5 ± 1.2 5.1 – 11.9 <0.01	2.9 ± 0.4 1.8 – 4.0 <0.01	5.6 ± 1.1 2.5 – 8.7 N.S.	6.4 ± 1.3 2.8 – 10.0 <0.01	36.9 ± 4.3 24.7 – 49.1 <0.01
E. Control group (nonneurologic cases) 15 cases	29.0 ± 1.6* 24.6 – 33.4†	5.1 ± 0.3 4.3 – 5.9	1.2 ± 0.07 1.0 – 1.4	4.0 ± 0.3 3.2 – 4.8	17.9 ± 0.7 16.0 – 19.8	24.0 ± 2.2 17.9 – 30.1

*Mean ± standard error (S.E.).

†Estimated range of population = mean ± tx (S.E.).

‡Probability at 0.01 level. N.S. = not significant.

i.e., 44 to 50 mg. per cent for the orcinol method[22] and 74 to 81 mg. per cent for the diphenylamine.[17, 18] Since the high order of specificity of the diphenylamine reaction for 2-desoxyhexoses and hexals has been stressed by several investigators,[16, 23] the difference in the results reported between the two procedures might either be due to the lesser specificity, or sensitivity, of the orcinol reagent or to the presence of unknown diphenylamine-reacting substances in both serum and C.S.F. In any event, significantly lower values for the C.S.F. total sialic acid are obtained for Group C, the amaurotic familial idiocy cases, whereas values almost twice the normal mean have been obtained for such disorders as amyotrophic lateral sclerosis, Parkinson's disease, and cerebral thrombosis, although the TP results are still in the normal range

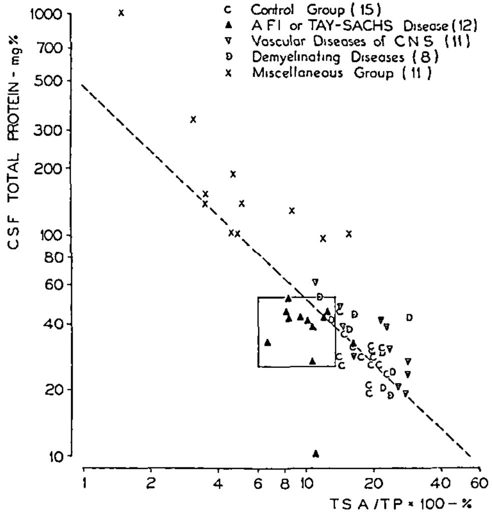

FIG. 1.—Variation of TSA/TP percentage ratio with increasing TP concentration of the C S F of 44 patients with various neurologic diseases and 15 control cases with nonneurologic disorders. The dotted line represents the median values of the control group

The relationship between the total sialic acid (TSA) and total protein (TP) of the C.S.F. for the 57 individual cases studied is illustrated graphically in Fig. 1 where the data are presented as a log-log plot of TP against TSA/TP ratio values. If the TSA/TP results varied in a manner exponential to the log of the TP values, then most of the points should fall along, or be adjacent to, the dotted line. While there is a general trend in the direction of lower TSA/TP ratios with higher TP values for most of the values, there are two notable exceptions among the groups of neurologic cases investigated. For

Group D, with extensive brain damage and high protein values, there is an increase in the total sialic acid that is disproportionate to that of the TP so that most points in this group lie above the line. In at least one instance the TSA/TP value approaches 1.0 per cent, the approximate relationship of the TSA/TP ratio in normal serum. Conversely, there appears to be a decrease in the total sialic acid in relation to the TP for the amaurotic familial idiocy group (Group C), many of the points falling into a definite region below the line (cf. Fig. 1, boxed area). The decrease in total sialic acid found in this disease is primarily due to the lower nonprotein-bound sialic acid values (see Table II).

The mean value of 1.2 ± 0.07 mg. per cent obtained for the protein-bound sialic acid fraction of the C.S.F. with the diphenylamine procedure is again considerably elevated above that found for the trichloroacetic acid precipitable fraction by Roboz and associates,[12] of 0.24 ± 0.3 mg. per cent. In a later publication Roboz and co-workers[13] employed alcohol precipitation at 4° C. for 24 hours as an improved procedure for protein precipitation from the C.S.F., requiring only 2.0 ml. per determination, prior to the application of the orcinol method for the determination of this fraction. They also reported increased protein-bound sialic acid levels in C.S.F. of patients with various neurologic disorders and the same normal value with alcohol precipitation as they had obtained with trichloroacetic acid precipitation. Uzman and Rumley[14] give a few individual values from which the protein-bound sialic acid obtained with trichloroacetic acid precipitation can be compared with the values obtained after dialysis for the same spinal fluids. The acid-precipitated protein-bound sialic acid varies from 0.22 to 0.47 mg. per cent whereas that found for the nondialyzable fraction varied from 0.45 to 0.81 mg. per cent for the same fluids. These findings have been confirmed in our laboratory and led us to question the completeness of protein precipitation by cold alcohol (4° C.) as a necessary step for the quantitative determination of the protein-bound sialic acid. It was found experimentally that only alcohol precipitation with heat at 65° C. for 30 minutes, as suggested by Südhof and associates,[24] gave results for C.S.F. protein-bound sialic acid that were comparable to those obtained with the nondialyzable protein fraction (see Table I). The procedure for the determination of this fraction, as described here, is less time consuming than those previously proposed and requires only 1.0 ml. of C.S.F. per determination.

Examination of the statistical data in Table II shows that there is a significant decrease in the protein-bound sialic acid values for Group C (amaurotic familial idiocy) as compared to controls. Only one case out of 12 in this group gave a value in excess of 1.0 mg. per cent. Markedly increased C.S.F. protein-bound sialic acid results are seen principally in patients of Group A, e.g., parkinsonism and cerebrovascular disorders.

The results given in Table III show some of the individual data obtained in various neurologic conditions in which the distribution of the sialic acid was studied simultaneously in C.S.F. and serum. The serum results again illustrate the unusually high values of the globulin sialic acid/total sialic acid percentage ratio found in amaurotic familial idiocy as compared to most other neurologic diseases.[18] These results also illustrate the wide range of values for total sialic acid, TSA/TP (per cent), and protein bound and nonprotein bound sialic acid which are found in pathologic spinal fluids. Again the cases of amaurotic familial idiocy, as a group (Table II), show significant decreases in the TSA/TP ratio and in the protein bound sialic acid values of the C.S.F. as compared to other neurologic disorders which may aid in the diagnosis and in the elucidation of the nature of this invariably fatal childhood disease.

TABLE III. INDIVIDUAL DATA OF BOTH CEREBROSPINAL FLUID AND SERUM SIALIC ACID DISTRIBUTION LEVELS IN PATIENTS WITH VARIOUS NEUROLOGIC DISEASES

		CEREBROSPINAL FLUID					SERUM		
PATIENT	DIAGNOSIS	TP (MG %)	TSA (MG %)	PBSA (MG %)	NPBSA (MG %)	TSA/TP %	TSA (MG %)	GSA (MG %)	GSA/TSA %
1 S.B.	Cerebral palsy	34.5	4.8	0.8	4.0	13.9	81.7	56.8	69.5
2 S.L.	Cerebral vascular disease with hemiplegia	47.7	6.7	1.5	5.2	14.0	76.8	43.6	56.7
3 I.S.	Postpolio	23.9	5.4	1.8	3.6	22.6	87.3	54.5	62.4
4 B.E.	Amyotrophic lateral sclerosis	43.0	12.4	1.7	10.7	28.8	—	—	—
5 J.B.	Multiple sclerosis	44.2	7.2	1.0	6.2	16.4	82.4	46.7	56.7
6 L.W.	Spina bifida	101.0	4.9	2.8	2.1	4.9	—	—	—
7 N.E.	Hydrocephalus, congenital	99.0	11.6	4.8	6.8	11.7	—	—	—
8 S.R.	Craniopharyngioma	138.0	4.8	2.6	2.2	3.5	—	—	—
9 J.M.	Schilder's disease	55.5	4.5	0.9	3.6	8.1	97.6	67.4	69.0
10 B.S.	Niemann-Pick disease	213.0	5.9	2.4	3.5	3.0	61.4	36.5	59.0
11 S.S.	A.F.I., infantile	25.0	3.2	0.8	2.4	12.7	101.0	67.4	67.0
12 V.R.	A.F.I., infantile	42.8	5.2	0.8	4.4	12.1	101.0	67.8	67.1
13 H.D.	A.F.I., infantile	47.5	4.1	1.1	3.0	8.6	107.0	74.6	69.7
14 K.S.	A.F.I., infantile	45.2	3.4	0.9	2.5	7.6	108.2	68.0	63.0
15 L.D.	A.F.I., juvenile	30.9	5.4	0.7	4.7	17.3	68.8	43.1	62.7
16 B.S.	Normal	42.8	4.8	1.3	3.5	11.1	61.7	33.7	54.6

Key to abbreviations: TP = total protein. TSA = total sialic acid. PBSA = protein bound sialic acid. NPBSA = nonprotein bound sialic acid. GSA = globulin sialic acid.

DISCUSSION

Studies of spinal fluid sialic acid distribution as applied to specimens obtained from *individual* patients have been few in number[12, 14] because of the lack of adequate quantitative methods requiring but small amounts of C.S.F. It was for this reason that the diphenylamine colorimetric reaction for serum sialic (or N-acetylneuraminic) acid previously published[17, 18] was employed in this investigation of the total, protein bound, and nonprotein bound sialic acid in C.S.F. Careful investigation of the characteristic purple diphenylamine color in serum by Hess and associates[16] has shown it to be characteristic of a 2-desoxyhexose, or hexal, structure and that this structure, or a common derivative of it, accounts for the adsorption maxima at 520 mμ. A desoxyketose structure in neuraminic acid (or its derivatives), as proposed by Gottschalk,[3-5] would also account for the color reaction of these substances with the diphenylamine reagent. Optical density measurements at or near this wave length of the diphenylamine color of the C.S.F. were, therefore, assumed to be a quantitative measure of their sialic acid content. The kind of sialic acid present in most biologic substances in which it is firmly bound to proteins is believed to be *N*-acetylneuraminic acid.[2, 3, 9] However, the nature of the neuraminic acid derivative present in C.S.F. which is dialyzable, and not bound to protein, has not as yet been established although it constitutes 60 to 80 per cent of the total sialic acid of this important biologic fluid.[14]

This investigation was undertaken in the belief that quantitative determinations of the sialic acid content of the C.S.F. would not only give diagnostic information but might also provide a clearer insight into the mechanism by which large amounts of sialic acid-containing gangliosides are deposited in brain gray matter in some lipoidoses.

SUMMARY

1. Methods are presented for the quantitative determination of the total sialic acid (TSA), protein-bound sialic acid, and nonprotein-bound sialic acid *of the cerebrospinal fluid (C.S.F.) which require a total of 1.5 ml. of fluid*, inclusive of the total protein (TP).

2. The colorimetric measurements are based on the relatively specific and sensitive diphenylamine (DPA) reaction which previously had been applied only to serum. Complete separation of the protein-bound from the nonprotein-bound sialic acid is achieved by use of alcohol precipitation with heat (30 min. at 65° C.).

3. Individual values were obtained for the C.S.F. of 42 patients with various neurologic diseases, as well as for a control group of 15 nonneurologic cases with normal TP values. Contrary to the findings in serum or tissues, 60 to 80 per cent of the TSA is present in a nonprotein-bound form.

4. Log-log plots of TSA/TP versus TP show a general trend in the direction of lower TSA/TP ratios with higher TP values for the control group (Group E) and for most neurologic conditions. Exceptions to this relationship were the cases with marked cerebral degeneration and high TP values, and the group of cases with amaurotic familial idiocy which showed highly significant differences by group statistics.

5. The general pathologic and diagnostic significance of sialic acid is discussed.

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THE EFFECT OF UREA ON CEREBROSPINAL FLUID PRESSURE IN MONKEYS BEFORE AND AFTER BILATERAL NEPHRECTOMY

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MANY hypertonic solutions have been used for reduction of cerebrospinal fluid pressure since the original work of Weed and McKibben.[1] Their undesirable features have markedly limited their use. The possibility of clinical application of urea was suggested by Fremont-Smith and Forbes in 1927[2]; however, their suggestion was not followed until 1954 when Javid and Settlage administered urea to human subjects.[3] More than 350 patients have received this agent for reduction of intracranial or intraocular pressure.[4, 5] About one hundred studies have been carried out in Rhesus monkeys.[6] The efficacy of urea has been compared with other hypertonic solutions as well as mercury and Diamox (acetazoleamide). Urea was found to be superior in effectiveness without producing adverse reactions. These findings added impetus in carrying out the following experiments.

The osmotic effect of hypertonic solutions and the inverse relationship between cerebrospinal fluid pressure and urinary output are well known.[1, 7] Experience with urea substantiates this point.[8-10] There is evidence that this reduction is presumably due to osmotic pressure change although it is possible that other factors are involved. The exact mechanism of action seems more complicated.[11] To determine the diuretic role of urea in lowering cerebrospinal fluid pressure, we investigated the effect of urea and other hypertonic agents in monkeys before and following nephrectomy.

MATERIALS AND METHODS

Five female Rhesus monkeys (*Macaca mulatta*) weighing 1.80 to 6.25 kilograms were the subjects. The animals were anesthetized with intramuscular pentobarbital, 42 mg. per kilogram body weight initial dose, and placed in a prone position. A cisternal needle was connected to a specially built open-end manometer consisting of polyethylene tubing (No. 100). Cerebrospinal fluid pressures were recorded every 5 minutes for 8 to 71 hours. Three monkeys received urea in 30 per cent concentration and 1 monkey sucrose in 50 per cent concentration; 1 received 50 per cent dextrose before and after nephrectomy followed by other hypertonic solutions. These solutions were given on an equimolar basis with one gram urea per kilogram body weight. A predetermined precise amount of urea, sucrose, and glucose was injected intravenously in divided doses every 30 seconds, in the course of 30 minutes to individual animals. In one study the duration of injection was 60 minutes. Rapid administration was also carried out as indicated in the figures.

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RESULTS

Fig 1 demonstrates an inverse relationship between cerebrospinal fluid pressure and volume of urine excreted following the administration of urea before and after unilateral nephrectomy.

It will be seen that maximum reduction of cerebrospinal fluid pressure corresponds with a maximum diuresis. However, the pressure drop is more pronounced and better sustained in the unilaterally nephrectomized monkey where diuresis is decreased.

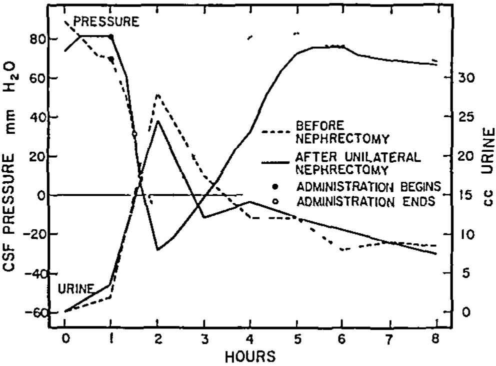

Fig 1.—Illustrated is the inverse relationship between cerebrospinal fluid pressure and urinary excretion before and after unilateral nephrectomy following administration of identical amounts of urea (30 per cent) in Monkey 104. Lowest cerebrospinal fluid pressure corresponds with maximum diuresis but pressure drop is greater and better sustained in unilaterally nephrectomized monkey.

Fig 2 shows the effect of urea when bilateral nephrectomy was done simultaneously. It can be seen that fall in cerebrospinal fluid pressure following nephrectomy was greater and more sustained. This monkey tolerated bilateral nephrectomy and was alert for 3 days, then failed rapidly and died on the fourth postoperative day.

In Fig 3 the cerebrospinal fluid pressure changes following unilateral and bilateral nephrectomy are compared with the pressure before nephrectomy. It will be noted that pressure drop following unilateral nephrectomy is greater than in the normal animal and the drop is still greater after bilateral nephrectomy. At the twenty eighth hour after the beginning of the experiment, a similar amount of urea was administered again. The pressure dropped promptly and reached -80 mm H_2O one hour after completion of urea administration. The animal was sacrificed 30 minutes later. Blood urea nitrogen was 182 *mg* per *100* ml, cerebrospinal fluid urea nitrogen was 125 mg per *100* ml. It is significant that, even in this bilaterally nephrectomized monkey, the discrepancy between the urea nitrogen level of blood and cerebrospinal fluid corresponds to what is always seen in a nonnephrectomized subject, i.e., during the first few hours after administration of urea, cerebrospinal fluid urea nitrogen levels lag behind that of blood.[3, 4]

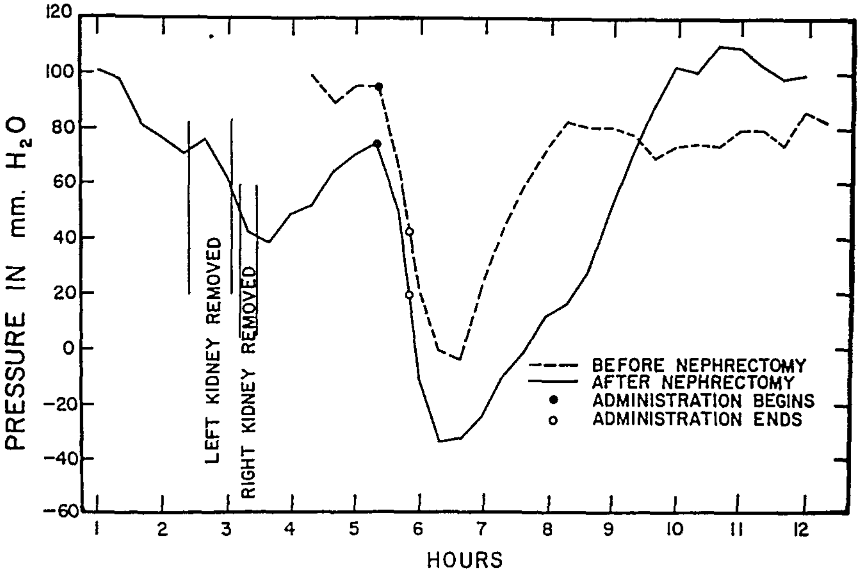

Fig. 2.—The cerebrospinal fluid pressure changes are demonstrated after urea administration in Monkey 88 before and following removal of both kidneys. Sufficient time was allowed following bilateral nephrectomy for cerebrospinal fluid pressure to return to prenephrectomy level before injection of urea.

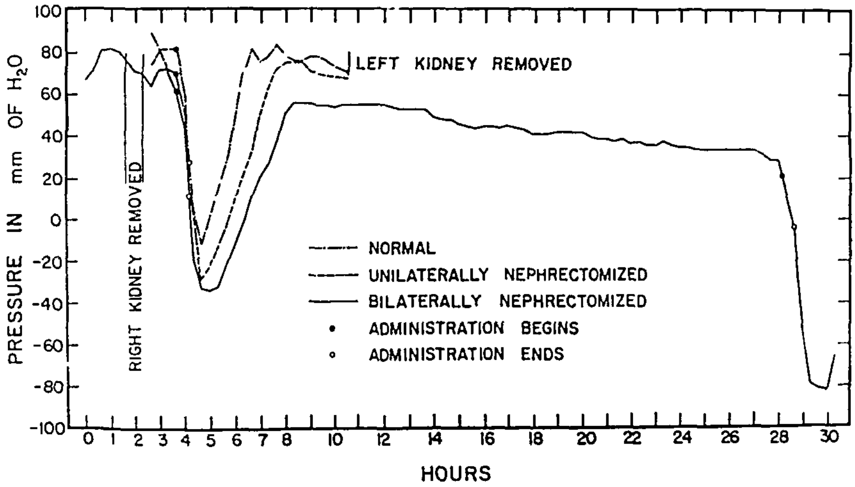

Fig. 3.—The cerebrospinal fluid pressure changes after urea administration in Monkey 104 before nephrectomy, following unilateral nephrectomy, and following bilateral nephrectomy are illustrated.

Fig. 4 illustrates pressure reduction following urea administration before and after nephrectomy. It can be seen that cerebrospinal fluid pressure reduction is more profound and better sustained after bilateral nephrectomy. At

23 hours postnephrectomy a similar dose of urea was injected rapidly, intraperitoneally, and produced a pressure drop to -89 mm H_2O At 29 hours post nephrectomy an identical amount was injected rapidly into the stomach and caused pressure to fall to 6 mm below atmospheric level The monkey died at 41 hours postnephrectomy

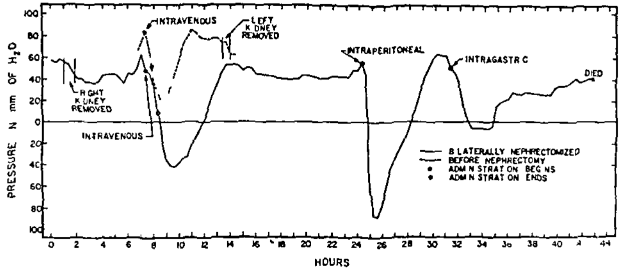

Fig 4—The cerebrospinal fluid pressure changes following urea administration before and after bilateral nephrectomy are shown for Monkey 92 The duration of injection after bilateral nephrectomy was 60 minutes

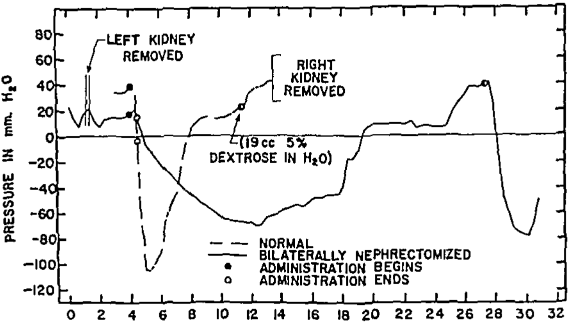

Fig 5—Monkey 113 Illustrated are cerebrospinal fluid pressure changes following 50 per cent sucrose administration equimolar with one gram per kilogram urea

Fig 5 shows pressure changes following 50 per cent sucrose administration, equimolar with one gram per kilogram urea The fall in cerebrospinal fluid pressure is slow after bilateral nephrectomy, but sustained for approximately 15 hours At 27 hours an identical amount of sucrose was injected rapidly There was a decrease of cerebrospinal fluid pressure to -78 mm H_2O The animal was sacrificed at 31 hours

Some additional observations were made in one monkey (No 109) following administration of 50 per cent dextrose equimolar with one gram per kilogram urea before and after nephrectomy It was noted that when dextrose was injected slowly during an interval of 30 minutes, reduction of cerebrospinal fluid pressure was minimal, but after bilateral nephrectomy the pressure drop was more marked. The lowering of cerebrospinal fluid pressure following rapid administration of dextrose (5 minutes) were identical before and after nephrectomy.

DISCUSSION

Findings in this report indicate that the effect of urea in reducing cerebrospinal fluid pressure is increased after nephrectomy. Thus, diuresis does not play an important role in lowering the intracranial pressure. Of interest are the following: the prompt and consistent fall in cerebrospinal fluid pressure in nephrectomized monkeys (Nos. 104 and 113) after similar amounts of urea and sucrose were repeatedly given, the effectiveness of urea in a nephrectomized monkey (No. 92)—whether urea was injected intravenously, intraperitoneally, or in the stomach.

The manner of fall in cerebrospinal fluid pressure in the nephrectomized monkey following sucrose, dextrose, and urea seems to be different. In the case of sucrose, there is the slow initial fall in cerebrospinal fluid pressure as compared to rapid fall with urea and dextrose. In the case of dextrose, the minimal discrepancy in cerebrospinal fluid reduction between pre- and postnephrectomy studies is probably due to the fact that this substance is metabolized, whereas sucrose and urea are not.

It would appear that prolonged maintenance of cerebrospinal fluid pressure reduction following urea administration in nephrectomized monkeys is due to prolonged osmotic pressure differential between blood and cerebrospinal fluid. The latter is due to the fact that urea cannot be eliminated via kidneys; consequently, it remains in a high concentration in the blood.

Clinically, whether inhibiting urinary output by use of drugs would increase the effectiveness of urea in reducing intracranial pressure, raises a question worthy of investigation.

SUMMARY

In order to determine the role of diuresis in reducing cerebrospinal fluid pressure following the administration of urea, a new approach is described. Bilateral nephrectomy was carried out in 5 Rhesus monkeys. In each instance, following administration of urea and other hypertonic solutions, the fall in cerebrospinal fluid pressure occurs and is better maintained in the absence of urinary excretion.

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LABORATORY METHODS

SPECTROPHOTOMETRIC DETERMINATION OF TOLBUTAMIDE IN PLASMA

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THE hypoglycemic agent tolbutamide (1-butyl-3-*p*-tolylsulfonylurea, Orinase*) can be quantitatively determined in serum or plasma by ultraviolet spectroscopy utilizing the intense absorption band at 228 mμ. Spingler and Kaiser[1] lyophilized the serum and extracted the tolbutamide from the dry residue with acidified ethyl acetate. A correction for the blank value of serum (no tolbutamide present) was made by obtaining a serum sample prior to tolbutamide administration, i.e., a zero hour sample. Forist and associates[2] used chloroform to extract the tolbutamide from plasma acidified to pH 4.5 to 4.8. A small amount of activated charcoal was used to decrease the blank value but a zero hour sample was recommended. Bladh and Nordén[3] also used the absorption band at 228 mμ to determine tolbutamide in serum. The serum was acidified with hydrochloric acid and chloroform was used as the extracting solvent. A column of calcium carbonate or sodium bicarbonate was used to reduce the blank value. A zero hour sample was specified.

Spingler[4] described a method based upon visible spectroscopy in which tolbutamide was extracted from acidified serum with amyl acetate. The tolbutamide was hydrolyzed to butylamine by heating at 150° C. for 5 minutes. The butylamine was then determined by adding 2,4-dinitrofluorobenzene and determining the absorbance at 380 mμ. A zero hour sample was not necessary.

A procedure for the quantitative determination of tolbutamide in plasma is described herein which is based upon the yellow color produced by the reaction of tolbutamide with Ehrlich's reagent (*p*-N-dimethylaminobenzaldehyde) under specified conditions. Since normal plasma constituents do not interfere, a zero hour sample is not necessary. The average recovery of added tolbutamide from human plasma was 100.4 per cent with a standard deviation of ± 5.2 per cent.

From the Department of Product Research and Development, The Upjohn Company, Kalamazoo, Mich.

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*Registered trademark, The Upjohn Company, Kalamazoo, Mich.

MATERIALS AND METHODS

'eagents—

. Phosphoric acid: 0.46 ml. of 85 per cent 0.30 M phosphoric acid diluted to 200 th distilled water.

. Phosphoric acid: 0.46 ml. of 85 per cent 0.15 M phosphoric acid diluted to 100 th distilled water.

. Chloroform: (Mallinckrodt) analytical reagent grade chloroform sealed in glass .

. Alcohol, 95 per cent ethanol (Commercial Solvents Corporation).

. Ehrlich's reagent solution: *p* N dimethylaminobenzaldehyde (Eastman Organic cals). Purified by dissolving in 95 per cent ethanol, decolorizing with carbon, and llizing by adding water; 100 ± 1 mg is dissolved in alcohol and diluted to 25 ml rown glass bottle. The solution is prepared immediately before using.

. Standard tolbutamide solution: A 0 7 to 0 8 Gm. quantity of the sodium salt of amide is weighed to the nearest milligram and transferred quantitatively to a 500 lumetric flask and made to volume with water. Various volumes, 10 to 80 ml, of lution are diluted to 500 ml. Sodium tolbutamide × 0 9247 = tolbutamide

pparatus.—

. *Separatory funnels:* Sixty milliliter separatory funnels fitted with Teflon stopcock were used for the extractions. After being washed with a synthetic detergent nsed with distilled water, they were further rinsed with alcohol. Pledgets of glass wool, previously washed two times with alcohol, were inserted in the stems funnels to remove droplets of water from the chloroform phase. Prior to being he funnels were rinsed with 10 ml chloroform which was allowed to filter through ass wool pledget.

. *Spectrophotometer:* A Beckman Model B spectrophotometer with 1 cm cuvettes *sed for the absorbance measurements*

Vacuum evaporator: A vacuum evaporator constructed in these laboratories was or the evaporation of the solvents.

Recommended Procedures—

1. Extraction: Duplicate 1 ml aliquots of oxalated plasma are mixed with 2 ml /30 phosphoric acid in separatory funnels containing 10 ml. chloroform The acidi- lasma, pH 4 5 to 4 8,[2] is extracted with the chloroform by gently rocking the tory funnel 20 times to and fro about an axis passing through the stopcock The form layer is slowly drained through the pledget of glass wool and collected in a round bottom flask which can be fitted to the vacuum evaporator. The extraction eated with two additional 10 ml portions of chloroform. The combined extracts are ntrated to dryness at room temperature Samples can be left at this stage

2. Color development: To one of the dry residues is added 2 ml. of Ehrlich's reagent on. To the other dry residue 2 ml of alcohol is added. To a clean flask 2 ml. of the h's reagent solution is added. All 3 are taken to dryness as above. (The second sample ts for the amount of color extracted from the plasma by the chloroform, the "chloro blank" [CB], and the third flask corrects for the amount of color due to the reagent, 'reagent blank" [RB]. See paragraph on calibration below and also the Discussion.) The dry residues are then heated for 2 5 hours in a water bath at 70° C. ± 1° C. To nt further development of color the flasks are then removed and cooled in running tap

3. Spectrophotometry. Ten milliliters of alcohol is added to the dry residues, and the bances (optical densities) of the solutions are measured at 395 mμ with alcohol in the nce cuvette within 8 minutes after the alcohol is added

4. Calibration: Aliquots of 1 ml. of the standard sodium tolbutamide solutions are added to plasma from a person not on tolbutamide therapy. One milliliter of M/15 phosphoric acid is added to each sample and the samples are carried through the above procedure. From the data a plot of absorbance versus concentration of tolbutamide can be prepared or alternatively the average absorptivity can be calculated. Beer's law was followed in the range 0 to 200 μg tolbutamide per milliter of plasma; absorbances of about 0.07 to 0.70 were obtained. A positive intercept in the calibration curve results from the use of alcohol in the reference cuvette instead of the unstable reagent blank.

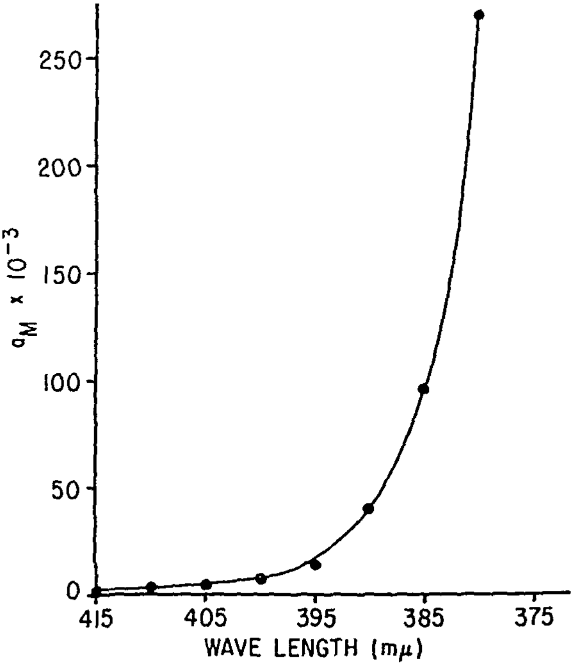

Fig. 1.—Absorption spectrum of the tolbutamide; Ehrlich's reagent chromogen (molar absorptivity calculated on the basis of the tolbutamide concentration of the final solution).

DISCUSSION

The color producing reaction between tolbutamide and Ehrlich's reagent could not be made to take place in solution although various types of solvents were used.[5] However, by heating the dry residue obtained as described reproducible results were obtained.

Fig. 1 is a plot of wave length versus molar absorptivity. Since the absorbance changes so rapidly with decreasing wave length it is important that the wave length scale of the instrument is accurate.

The color does not fade rapidly. There is no fading during the first 8 minutes. A 4 per cent fading occurred during the next 22 minutes.

The customary reagent blank, i.e., no plasma plus the reagent, is not used in the reference cuvette because this would necessitate a reagent blank for each 3 determinations as the color of the reagent blank is also unstable. The absorbance of the reagent blank was determined each time a new Ehrlich's reagent solution was made. The absorbance of the reagent blank is fairly reproducible (see Table I).

In a determination such as this the blank value of plasma (no tolbutamide present) is due to two factors. One is the color extracted from the plasma by the chloroform (the chloroform blank) and the other is the presence of materials extracted by the chloroform which react with the Ehrlich's reagent to produce color (the plasma blank). The first factor can be evaluated readily by carrying a sample through the procedure and not adding the color producing reagent. For the evaluation of the second factor a zero hour sample is necessary.

TABLE I. EVALUATION OF BLANK VALUES

PLASMA SAMPLE	A_0 PLASMA (ETC.) + EHRLICH'S	REAGENT BLANK NO PLASMA (ETC.) + EHRLICH'S	$CHCl_3$ BLANK PLASMA (ETC.) + NO EHRLICH'S		PLASMA BLANK A_0 − (RB + CB)	
			A_{803}	TOLBUTAMIDE EQUIVALENT*	A_{803}	TOLBUTAMIDE EQUIVALENT*
ST	0.099	0.086	0.010	3 μg./ml.	0.003	1 μg./ml.
DEG	0.093	0.077	0.010	3	0.006	2
RCA†	0.089	0.078	0.005	2	0.006	2
PO	0.083	0.076	0.008	3	−0.001	0
DWA	0.084	0.075	0.002	1	0.007	2
ERB	0.082	0.073	0.001	0	0.008	2
POL	0.090	0.081	0.006	2	0.003	1
PT	0.085	0.076	0.005	2	0.004	1
CH	0.090	0.085	0.008	2	−0.003	−1
RM	0.088	0.083	0.007	2	−0.002	1
			Average	2.3		0.9‡

*Absorbance of 0.001 = 0.3 μg. tolbutamide per milliliter of plasma.
†Diabetic patient.
‡Average correction for zero hour sample.

Then the plasma blank, PB = total absorbance (A_0) − reagent blank (RB) − chloroform blank (CB). The data in Table I indicate a plasma blank equivalent to about 1 μg. tolbutamide per milliliter. Since plasma levels of tolbutamide range from 30 to 200 μg. per milliliter for individuals receiving the drug[1, 3, 4] the plasma blank may be ignored, thus, the need for a zero hour sample is obviated.

TABLE II. RECOVERY OF ADDED TOLBUTAMIDE FROM HUMAN PLASMA
(Micrograms per milliliter of plasma)

ADDED	FOUND	PER CENT RECOVERY	AVERAGE
83	89, 90, 81	107.2 108.4 97.5	104.5
111	115, 116, 115	103.6 104.5 103.6	103.9
138	148, 142, 135	107.2 102.9 97.8	102.6
166	173, 170, 151	104.2 102.4 91.0	99.2
194	191, 193, 187	98.5 99.5 96.4	98.1
222	210, 208, 208	94.6 93.7 93.7	94.0
Average ± standard deviation (n = 18) 100.4 ± 5.2 per cent			

The recovery of added tolbutamide to human plasma is shown in Table II. The average recovery is 100.4 per cent with a standard deviation of 5.2 per cent. The values have been corrected for the chloroform blank but not for the plasma blank. That is, the data are comparable to the situation wherein a zero hour sample is not possible.

Hemolysis was not a problem in these studies.

SUMMARY

A procedure is described for the determination of tolbutamide in human plasma. The acidified plasma is extracted with chloroform, the chloroform extract is taken to dryness, an alcoholic solution of Ehrlich's reagent (*p*-N-dimethylaminobenzaldehyde) is added and the solution taken to dryness. The dry residue is heated at 70° C. for 2.5 hours. Alcohol is added and the absorbance of the solution is measured at 395 mμ. The average recovery of tolbutamide from human plasma has been 100.4 per cent with a standard deviation of 5.2 per cent. A zero hour sample is not necessary.

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AN IMPROVEMENT OF THE METHOD FOR DETERMINATION OF PLASMA AND URINE HEMOGLOBIN

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THE benzidine method for the measurement of small amounts of hemoglobin[1] utilizes undiluted specimens when the concentration of hemoglobin is less than 30 mg. per 100 ml. With higher concentrations, appropriate dilution of the unknown is necessary, a process which consumes time and may reduce accuracy. The precise measurement of a very small volume would permit the use of undiluted material even when the concentration of hemoglobin is relatively high. The purpose of this communication is to describe a modification of the "Dacie pipette"[2] which may be used for the measurement of volumes as small as 1 lambda. This permits the use of undiluted specimens with concentrations of hemoglobin as high as 400 mg. per 100 ml.

METHODS

The instrument consists of a Pasteur pipette used to brace a small length of capillary tubing. This tubing is broken to a desired length—to correspond to the approximate volume to be measured—and is inserted into the tip of the pipette and fixed there with a bit of modeling clay. In the rubber bulb of the pipette, a hole is made about 2 mm. in diameter (Fig. 1). The reagents used are those employed for the benzidine method: an acid solution of benzidine, hydrogen peroxide, and acetic acid.[1]

The tubing is filled by holding the pipette horizontal and touching the tip of the capillary to the surface of the liquid to be measured. This means that the test tube of liquid must be tipped to bring the liquid to the rim. The capillary is emptied by holding the pipette vertical and permitting the liquid to run into the benzidine solution. To empty it completely, the hole in the rubber bulb is covered with the finger tip, and the bulb is gently squeezed and released to empty and rinse the capillary. The hemoglobin benzidine mixture is now treated with hydrogen peroxide in the usual manner[1] and the intensity of color which develops is measured photometrically. It is unnecessary to know the exact volume of the capillary, because the same capillary is used to measure an identical amount of a standard hemoglobin solution and the results of the standard and the unknown are compared to derive the concentration of hemoglobin in the unknown. The same capillary may be used to measure an entire series of unknowns if, after each measurement, it is rinsed first with water and then with acetone.

The standard solutions of hemoglobin are prepared first by measuring the concentration of hemoglobin in a sample of whole blood by the cyanmethemoglobin method.[3] The red cells are then hemolyzed by freezing and thawing and the blood is diluted with a mixture of saline and glycerol (equal parts) to achieve several desired concentrations. 20, 80, and 200 mg. of hemoglobin per 100 ml. provide a satisfactory covering of the concentrations measured by this method. These glycerolized preparations are stable and may be retained and used for many months.[4]

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RESULTS

Several trials were undertaken to establish the limitations of the method. Table I demonstrates the reproducibility of results when two pipettes were used to measure repeatedly a single sample of plasma. There is less than 1 per cent variability. Table II demonstrates the results of a "blind" experiment. A sample of plasma was stained by adding to it a small amount of gentian violet so that the operator could not estimate by eye the hemoglobin concentration. Then the sample was divided into 6 tubes and a small amount of hemoglobin was added to each, measuring it to achieve concentrations of approximately 40, 60, 80, 120, and 200 mg. hemoglobin per 100 ml. The concentration of hemoglobin of each specimen was measured on 3 different days and the results are shown in Table II. In each instance the results agree within about 5 per cent.

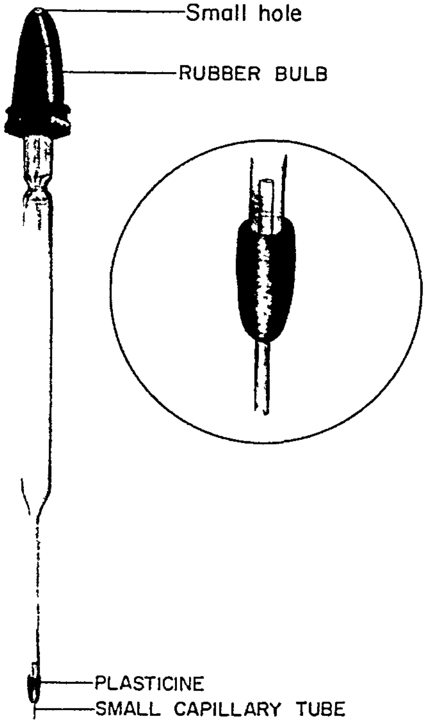

Fig. 1.—A modification of the Dacie pipette to enable measurement of small volumes.

It is possible to encompass the entire range of plasma hemoglobins using 3 or 4 of these little pippettes, each a different size. Table III demonstrates how this was done with 3 pipettes, the approximate volumes of which were 20, 5, and 1.5 lambda. For any given unknown the proper pipette is selected so that the reading of transmittance on the photometer scale lies within the range of best accuracy of the instrument, which is usually between 20 and 80 per cent.

TABLE I REPLICATE DETERMINATIONS OF PLASMA HEMOGLOBIN PERFORMED ON A SAMPLE OF PLASMA USING TWO PIPETTES

TUBE	PHOTOMETER READING	MG /100 ML	TUBE	PHOTOMETER READING	MG /100 ML
1	28^{3}	87.2	1	31	87.0
2	29	87.0	2	31	87.0
3	29	87.0	3	31	87.0
4	29	87.0	4	31	87.0
5	28^{3}	87.2	5	31	87.0
6	28^{3}	87.2	6	31	87.0
7	29	87.0	7	31	87.0
8	29	87.0	8	31	87.0
9	29	87.0	9	31	87.0
10	28^{3}	87.2	10	31^{7}	86.8
11	29	87.0	11	30^{3}	87.5
12	29	87.0	12	31	87.0
13	29	87.0	13	31	87.0
14	29	87.0	14	31	87.0
15	29	87.0	15	31^{7}	86.8
Standard	56	40.0	Standard	58^{7}	40.0

TABLE II "BLIND" DETERMINATION OF PLASMA HEMOGLOBIN IN STAINED PLASMAS CONTAINING APPROXIMATELY 40, 60, 80, 120, 160, AND 200 MG PER 100 ML

	TUBE	MG PER CENT		TUBE	MG PER CENT		TUBE	MG PER CENT
Nov 18	1	39	Nov 20	1	38	Nov 25	1	42
	2	66		2	62		2	57
	3	86		3	90		3	90
	4	114		4	115		4	118
	5	168		5	173		5	173
	6	201		6	201		6	201

TABLE III DEMONSTRATING THE USE OF PIPETTES OF SEVERAL SIZES TO PERMIT MEASUREMENT OF A WIDE RANGE OF PLASMA HEMOGLOBIN CONCENTRATIONS

PIPETTE 1 APPROXIMATE VOLUME 20 LAMBDA		PIPETTE 2 APPROXIMATE VOLUME 5 LAMBDA		PIPETTE 3 APPROXIMATE VOLUME 1.5 LAMBDA	
PLASMA Hb CONCENTRATION (MG /100 ML)	PHOTOMETER READING	PLASMA Hb CONCENTRATION (MG /100 ML)	PHOTOMETER READING	PLASMA Hb CONCENTRATION (MG /100 ML)	PHOTOMETER READING
0	0	20	79	80	76
10	54	40	57	100	65
20	32	60	44	120	64
30	18	80	35	160	58
--	-	100	27	200	52
-	--	120	21	300	33
--	--	-	-	350	29
--	-	--	-	400	25
--	-	--	--	450	21

Occasionally a specimen may be encountered where the concentration of hemoglobin exceeds 400 mg Such specimens may be diluted and measured by the benzidine method or, better, the concentration of hemoglobin may be measured directly by the cyanmethemoglobin technique,[3] taking care to add a sufficiently large volume of the unknown and correcting the results accordingly

SUMMARY

A modification of the "Dacie pipette" is described which permits the measurement of small volumes of plasma or urine This permits the use of the

RESULTS

Several trials were undertaken to establish the limitations of the method. Table I demonstrates the reproducibility of results when two pipettes were used to measure repeatedly a single sample of plasma. There is less than 1 per cent variability. Table II demonstrates the results of a "blind" experiment. A sample of plasma was stained by adding to it a small amount of gentian violet so that the operator could not estimate by eye the hemoglobin concentration.

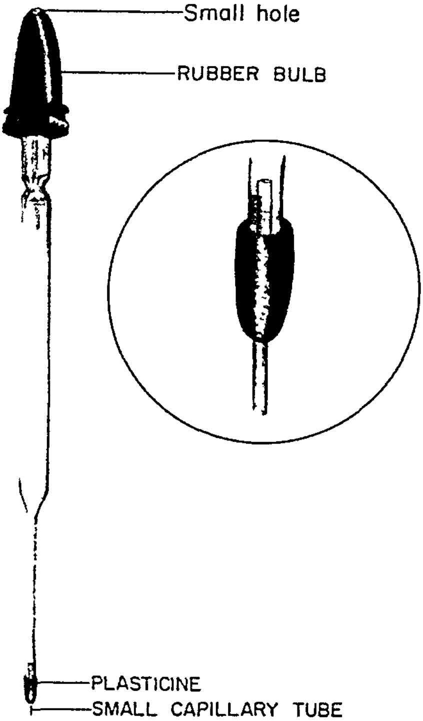

Fig. 1.—A modification of the Dacie pipette to enable measurement of small volumes.

Then the sample was divided into 6 tubes and a small amount of hemoglobin was added to each, measuring it to achieve concentrations of approximately 40, 60, 80, 120, and 200 mg. hemoglobin per 100 ml. The concentration of hemoglobin of each specimen was measured on 3 different days and the results are shown in Table II. In each instance the results agree within about 5 per cent.

It is possible to encompass the entire range of plasma hemoglobins using 3 or 4 of these little pippettes, each a different size. Table III demonstrates how this was done with 3 pipettes, the approximate volumes of which were 20, 5, and 1.5 lambda. For any given unknown the proper pipette is selected so that the reading of transmittance on the photometer scale lies within the range of best accuracy of the instrument, which is usually between 20 and 80 per cent.

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EARLY AND LATE HEMODYNAMIC EFFECTS OF MITRAL COMMISSUROTOMY

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THE hemodynamic changes in the immediate postoperative period and short term following mitral commissurotomy have been reported by several authors.[1-6] An excellent long-term clinical study has been published by Ellis and Harken[7]; however, with two exceptions,[8, 9] published reports on the circulatory dynamics in the long-term postoperative period have been few and rather limited.[10-12]

The patients constituting the series to be dealt with herein were the first 12 among those subjected to mitral commissurotomy during the period April, 1951, through October, 1952, who accepted right heart catheterization 3 or more times and whose follow-ups have been extended 4½ to 6 years from the dates of operation. A standard technique of catheterization was used.[1, 13] Generally, the occasions were 1 week before commissurotomy, 3 weeks after (range 10 to 90 days), and 1 year after (range 8 to 30 months).

A brief summary of the hemodynamic data obtained has appeared elsewhere.[14] This paper is to present the complete data on these 12 patients, fully documenting the earlier statements concerning them, and to offer an analysis of the data.

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A portion of this material is derived from a thesis submitted by Dr. Tompkins to the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Master of Science in Medicine.

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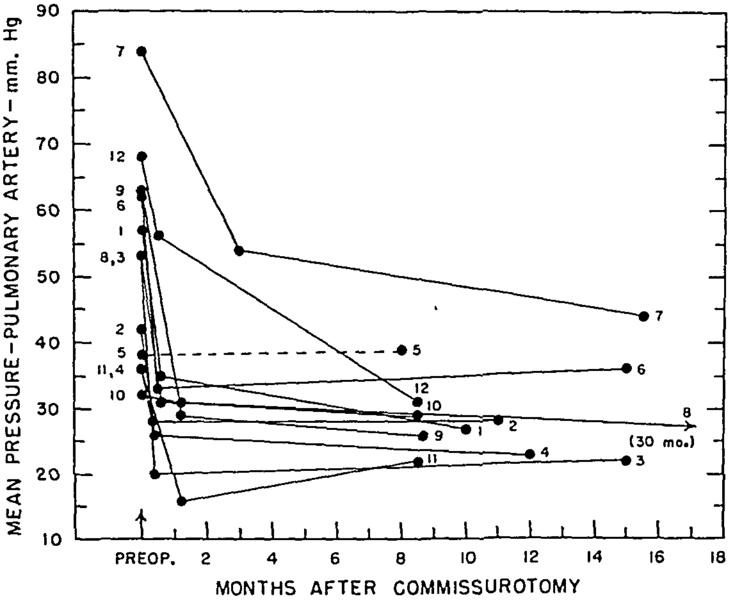


FIG. 1.—Pulmonary artery mean pressure at rest, before mitral commissurotomy, and in follow-up studies. The decrease after commissurotomy was consistent and the lower postoperative pulmonary artery pressure was maintained essentially unchanged through the rest of the period shown. The numerals designate the individual patients listed in Table I. The broken lines connect values for which corresponding early postoperative data were not obtained.

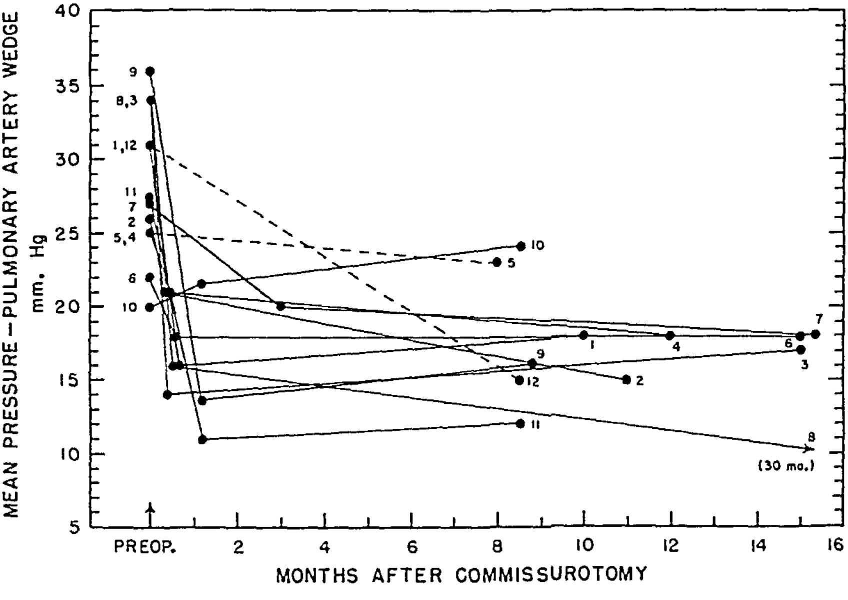
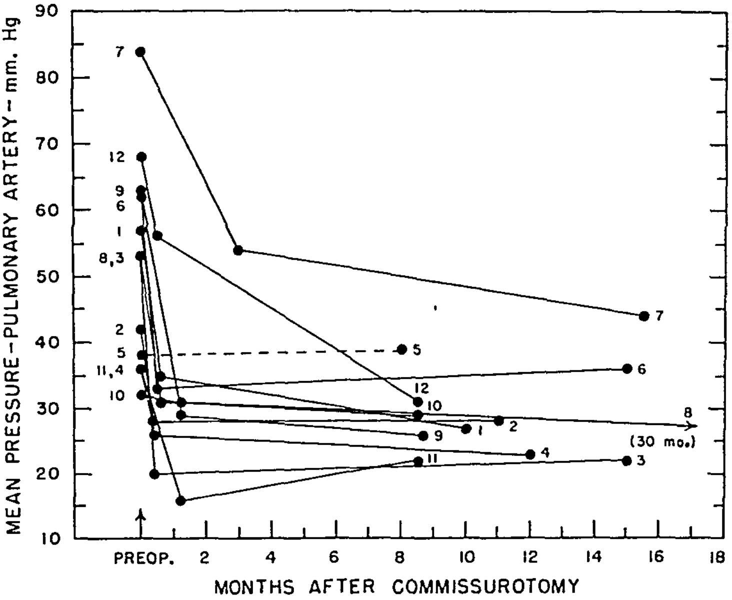


FIG. 2.—Pulmonary artery wedge pressure at rest, before mitral commissurotomy, and in follow-up studies. After the early postoperative decrease the wedge pressure remained practically unchanged through the rest of the period shown. (Details are explained in the legend for Fig. 1.)



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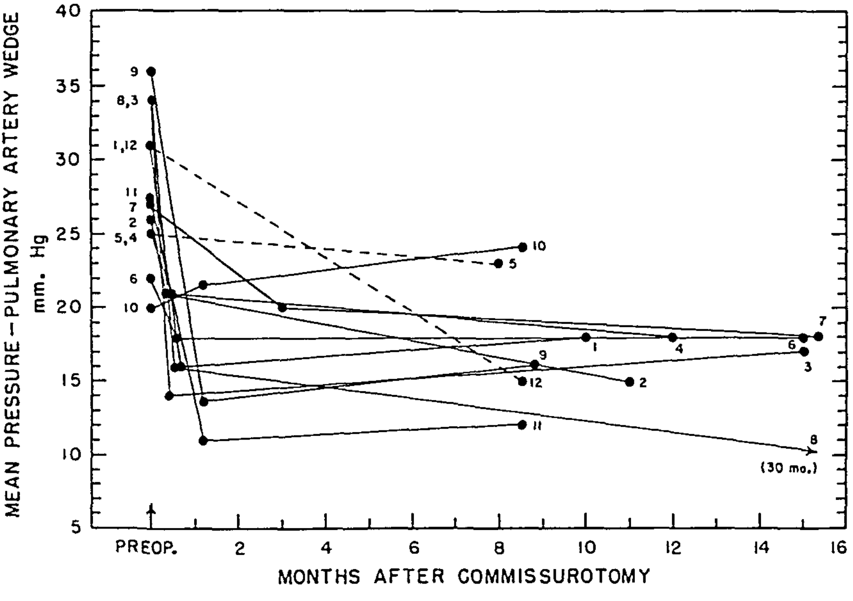
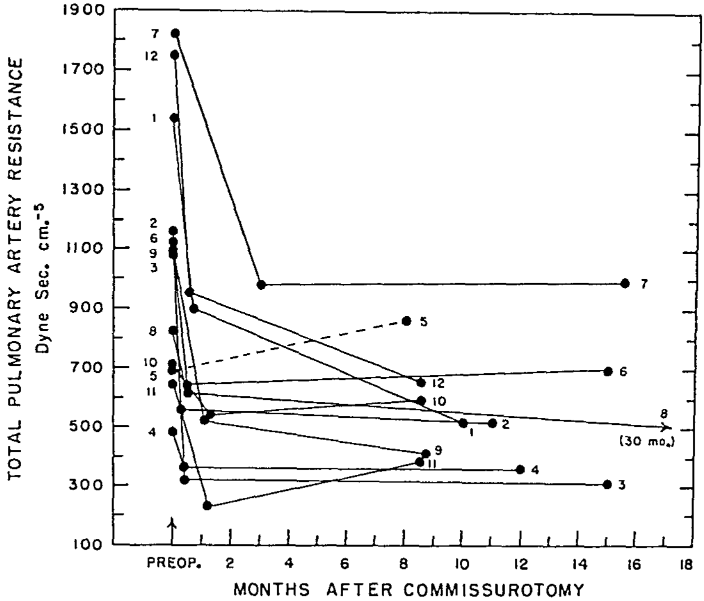


Fig. 2.—Pulmonary artery wedge pressure at rest, before mitral commissurotomy, and in follow-up studies. After the early postoperative decrease the wedge pressure remained practically unchanged through the rest of the period shown. (Details are explained in the legend for Fig. 1.)

1 Week Before Commissurotomy, in the Early Postoperative Period, and Postoperative Follow-Up

Pulmonary Artery		Right Atrial Mean Pressure (mm Hg)	Vascular Resistance Dyne Sec Cm^{-5}		Mitral Valve Area Index (cm^2)	Breathing 99.5 Per Cent Oxygen	
Mean Pressure (mm Hg)	Mean Wedge Pressure (mm Hg)		TPR*	PAR†		Cardiac Index (L/min/m^2)	Pulmonary Artery Mean Pressure (mm Hg)
57	31	7	1,540	700	0.4	1.7	47
63	42	—	1,710	570			
35	16	5	900	490	0.7	1.9	32
55	22	—	1,170	550			
27	28	5	520	100	0.9	1.8	30
56	29	—	930	350			
42	26	6	1,130	430	0.4	1.5	40
67	—	—	1,570	—			
28	21	5	560	140	0.7	2.1	23
38	27	—	680	200			
28	15	8	520	150	0.8	2.1	30
30	26	—	530	180			
53	34	4	1,090	390	0.7	2.8	35
62	—	—	1,030	—			
20	14	3	320	100	1.6	2.8	18
44	34	—	430	130			
22	17	9	270	70	1.8	3.6	19
43	40	—	460	30			
36	25	4	490	150	1.5	2.7	33
47	39	—	570	100			
26	21	7	370	70	1.4	3.0	23
42	—	—	410	—			
23	18	3	370	80	1.5	2.3	42
44	28	—	430	50			
38	25	10	690	200	0.7	2.3	42
—	—	—	—	—			
—	—	23	—	—	—	—	—
—	—	—	—	—			
39	23	12	870	360	0.6	2.2	42
73	34	—	1,080	580			
62	22	4	1,130	730	1.1	2.3	48
—	—	—	—	—			
33	18	7	650	290	1.2	2.6	35
—	—	—	—	—			
36	18	3	700	470	1.1	—	—
68	27	—	1,100	670			
68	24	6	1,500	1,000	0.7	3.1	56
84	27	8	1,830	1,240	0.6	2.0	59
112	—	—	3,130	1,960			
54	20	6	990	620	0.9	—	46
113	—	—	1,800	—			
44	18	1	1,000	590	0.8	2.6	40
108	43	—	1,730	1,040			
53	34	9	610	290	0.8	2.2	47
—	—	—	—	—			
31	16	9	710	340	0.9	2.1	25
—	—	—	—	—			
26	10	4	510	310	1.5	2.4	18
43	26	—	470	190			



monary artery resistance at rest, before mitral commissurotomy, and in The total pulmonary vascular resistance decreased consistently and the [illegible] values were *maintained within the limits of error of such determinations* through the rest of the period shown (Details are explained in the legend for Fig 1)

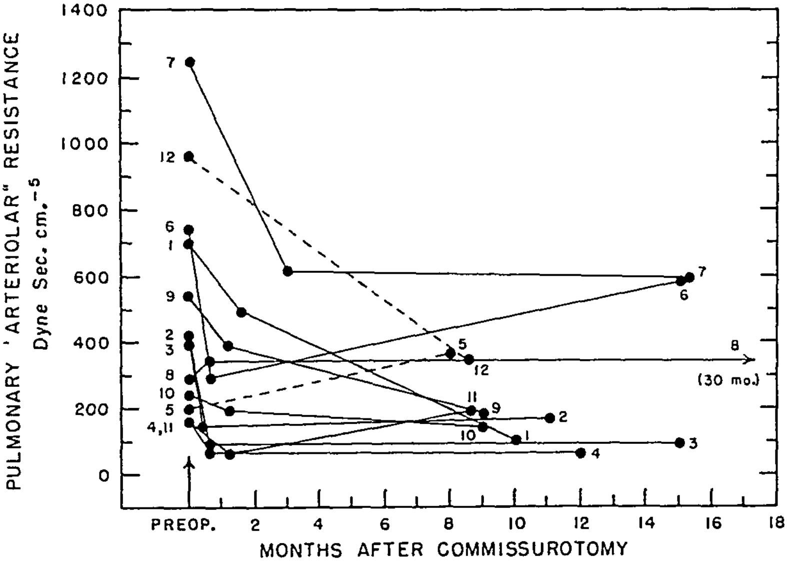


Fig 4 — nce at rest, before mitral commissurotomy, and in follow-up stud values were generally maintained within the limits of error for su ie rest of the period shown. (Details are explained in the legend for Fig 1)

In these 12 cases pulmonary arteriolar resistance comprised roughly half of the total resistance across the lesser circulation. The improvement with respect to this variable was proportional to the reduction in total pulmonary resistance (Fig. 4).

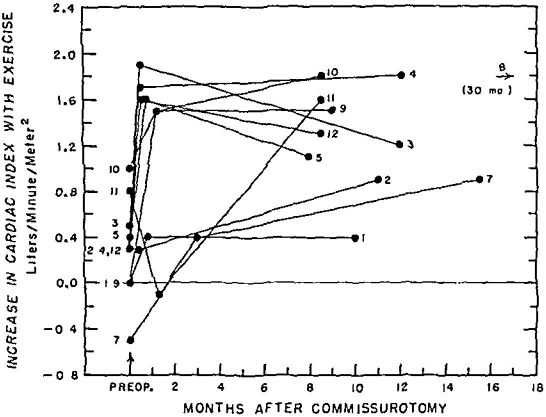


Fig. 5.—Changes in cardiac output with exercise before mitral commissurotomy and in follow-up studies. The improvement in the increase of cardiac output with exercise was well maintained during the period shown. (Details are explained in the legend for Fig. 1.)

Cardiac Index During Rest and Exercise.—The normal cardiac index at rest has been given by Cournand[15] as 3.1 to 3.8 L./min./M.^{2} The figures for healthy subjects studied[16] in this laboratory are 2.7 to 4.3 L./min./M.^{2} with a mean increase on a standard form of exercise of 1.0 to 3.2 L./min./M.^{2} Of the 12 patients in the present series, 8 showed a rise in the resting cardiac index postoperatively (Fig. 5). Six of these were patients whose preoperative index was subnormal, that is, below 2.7 L./min./M.^{2} (Table I). Preoperatively, the 10 patients from whom the comparative data were obtained could increase their resting cardiac index by an average of only 12 per cent (310 ml./min./M.^{2}) in response to exercise. In the early postoperative period they were able to increase it by 33 per cent, and 8 to 30 months postoperatively the entire series of 12 produced an average increase of 48 per cent. The absolute increase in response to exercise correlated well with the degree of clinical improvement (Fig. 6). At 8 to 30 months postoperatively the 7 patients who had improved from functional Class III or IV to Class I were able to increase their cardiac index above the resting level by 1.1 L./min./M.^{2} or more, for an average increase of 1.5 L./min./M.^{2} The 5 patients who had improved from Class III to Class II were able to increase their index by an average of 0.9 L./min./M.^{2}

Stroke Index.—In healthy subjects, under the conditions of exercise in this laboratory, cardiac output is increased primarily through an increase in heart

TABLE I—

CASE	TIME OF STUDY	AGE AND SEX	N. Y. HEART GROUP	STATE	SURFACE AREA (M.^{2})	HEART RATE	OXYGEN CONSUMPTION (ML./MIN.-/M.^{2})	CARDIAC INDEX (L./MIN./M.^{2})
9	Preop.	32	IV d	R	1.52	96	142	3.0
		F		Ex		120	214	3.0
	Postop. 5 wk.		II c	R	1.46	64	133	3.0
				Ex		102	348	4.5
	Postop. 9 mo.		I b	R	1.52	66	143	3.3
				Ex		112	452	4.8
	Postop. 58 mo.		II b	R	1.56	150‡	149	2.9
				Ex				
10	Preop.	42	III c	R	1.56	80	113	2.3
		F		Ex		93	244	3.3
	Postop. 5 wk.		II c	R	1.56	93	120	3.0
				Ex		126	339	4.5
	Postop. 8½ mo.		I b	R	1.62	76	115	2.4
				Ex		138	405	4.2
11	Preop.	38	III c	R	1.30	76	138	3.5
		F		Ex		130	389	4.3
	Postop. 5 wk.		II b	R	1.30	90	170	4.2
				Ex		114	338	4.1
	Postop. 8½ mo.		II b	R	1.35	96	135	3.4
				Ex		124	425	5.0
12	Preop.	31	III d	R	1.45	80	135	2.1
		F		Ex		114	262	2.4
	Postop. 16 days		II c	R	1.40	110	157	3.3
				Ex		124	388	4.9
	Postop. 8½ mo.		I b	R	1.49	72	126	2.5
				Ex		132	391	3.8
	Postop. 47 mo.		II c	R	1.50	88	138	2.4
				Ex			333	2.7
Averages								
	Preoperative (1 week)			R			135	2.6
				Ex			278	2.8
	Postoperative (10 to 90 days)			R			136	2.9
				Ex			311	3.9
	Postoperative (8 to 30 months)			R			128	2.7
				Ex			390	4.0

R = resting state; Ex = on exercise.
*TPR = total pulmonary resistance.
†PAR = pulmonary arteriolar resistance.
‡Atrial fibrillation occurred.

artery wedge pressure at rest or on exercise. The clinical improvement was very satisfactory, however, and a significant increase in cardiac output during exercise was demonstrable. With regard to the rise in wedge pressure with exercise, the comparative data are available on 5 patients before operation and 8½ to 12 months after operation. The average mean wedge pressure at rest for these 5 patients was 27 mm. Hg (range 20 to 31) preoperatively with a rise to 38 (range 29 to 42) on exercise. At determinations 8½ to 12 months postoperatively, the average mean wedge pressure at rest was 19 (range 12 to 28) with a rise to 30 (range 20 to 43) on exercise. Thus, the increase in

Cont'd

PULMONARY ARTERY MEAN PRESSURE (MM Hg)	PULMONARY ARTERY MEAN WEDGE PRESSURE (MM Hg)	RIGHT ATRIAL MEAN PRESSURE (MM Hg)	VASCULAR RESISTANCE DYNE SEC CM⁻⁵ TPR*	VASCULAR RESISTANCE DYNE SEC CM⁻⁵ PAR†	MITRAL VALVE AREA INDEX (CM²)	BREATHING 99.5 PER CENT OXYGEN: CARDIAC INDEX (L/MIN/M²)	BREATHING 99.5 PER CENT OXYGEN: PULMONARY ARTERY MEAN PRESSURE (MM Hg)
63	36	5	1,100	540	0.7	3.6	48
67	—	—	1,160	—			
29	14	2	530	280	1.2	—	24
59	28	—	730	380			
26	16	2	400	160	2.0	2.7	20
50	—	—	500	—			
49	37	9	870	210	0.7	—	—
32	20	4	710	240	0.8	2.4	24
44	38	—	690	90			
31	22	9	540	150	1.2	—	21
59	41	—	670	210			
29	24	7	600	100	0.8	—	—
75	43	—	890	380			
36	28	2	640	150	0.9	3.0	30
65	29	—	930	510			
16	11	4	240	70	2.2	3.4	12
25	19	—	380	90			
22	12	3	380	180	1.7	3.5	15
34	20	—	400	40			
68	31	9	1,750	950	0.5	—	59
89	42	—	2,090	1,100			
56	—	7	950	—	—	3.6	43
65	—	—	770	—			
31	15	2	660	340	1.1	2.3	27
64	29	—	900	490			
64	35	9	1,420	640	0.5	2.4	56
81	39	—	1,620	840			
52	28		1,060	500	0.8	2.4	43
68	38		1,430	720			
33	17		610	260	1.2	2.7	27
56	29		780	260			
29	18		570	240	1.2	2.6	28
58	31		790	360			

the mean wedge pressure on exercise 8½ to 12 months postoperatively was rather closely proportionate to the preoperative increase However, absolute values were substantially lower, so in 4 of the 5 patients the postoperative wedge pressure during exercise was approximately equal to or considerably lower than the preoperative value during rest

Calculated Pulmonary Vascular Resistance—At rest and during exercise the calculated total pulmonary resistance was reduced by an average of approximately 45 per cent in the immediate postoperative period, and although individual patients showed increases or decreases on restudy at 8 to 30 months after operation, the major portion of the large initial reduction was uniformly maintained (Fig 3)

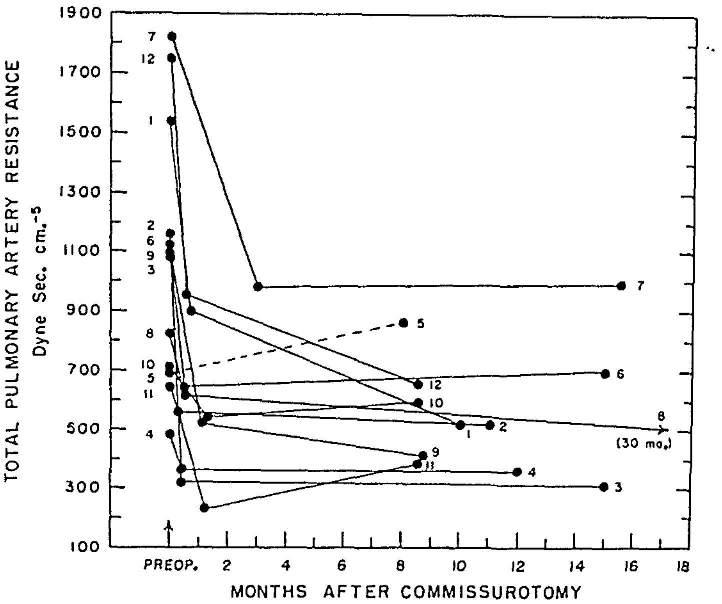


Fig. 3.—Total pulmonary artery resistance at rest, before mitral commissurotomy, and in follow-up studies. The total pulmonary vascular resistance decreased consistently and the lower postoperative values were maintained within the limits of error of such determinations through the rest of the period shown. (Details are explained in the legend for Fig. 1.)

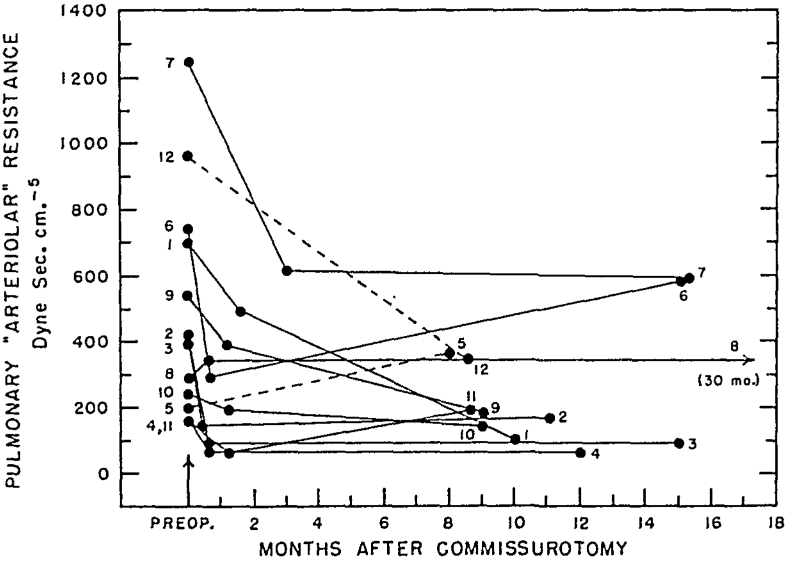


Fig. 4.—Pulmonary arteriolar resistance at rest, before mitral commissurotomy, and in follow-up studies. The lower postoperative values were generally maintained within the limits of error for such determinations through the rest of the period shown. (Details are explained in the legend for Fig. 1.)

In these 12 cases pulmonary arteriolar resistance comprised roughly half of the total resistance across the lesser circulation. The improvement with respect to this variable was proportional to the reduction in total pulmonary resistance (Fig. 4).

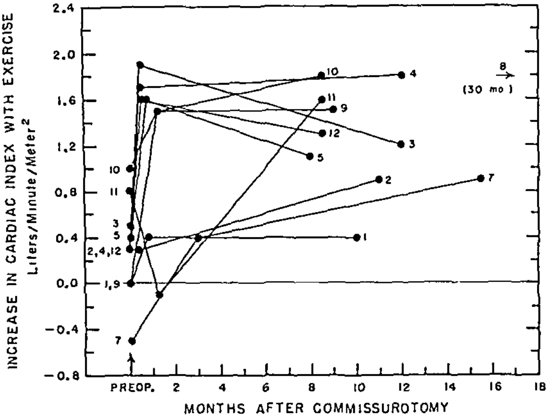


Fig. 5.—Changes in cardiac output with exercise before mitral commissurotomy and in follow-up studies. The improvement in the increase of cardiac output with exercise was well maintained during the period shown. (Details are explained in the legend for Fig. 1.)

Cardiac Index During Rest and Exercise.—The normal cardiac index at rest has been given by Cournand[15] as 3.1 to 3.8 L./min./M.^{2} The figures for healthy subjects studied[16] in this laboratory are 2.7 to 4.3 L./min./M.^{2} with a mean increase on a standard form of exercise of 1.0 to 3.2 L./min./M.^{2} Of the 12 patients in the present series, 8 showed a rise in the resting cardiac index postoperatively (Fig. 5). Six of these were patients whose preoperative index was subnormal, that is, below 2.7 L./min./M.^{2} (Table I). Preoperatively, the 10 patients from whom the comparative data were obtained could increase their resting cardiac index by an average of only 12 per cent (310 ml./min./M.^{2}) in response to exercise. In the early postoperative period they were able to increase it by 33 per cent, and 8 to 30 months postoperatively the entire series of 12 produced an average increase of 48 per cent. The absolute increase in response to exercise correlated well with the degree of clinical improvement (Fig. 6). At 8 to 30 months postoperatively the 7 patients who had improved from functional Class III or IV to Class I were able to increase their cardiac index above the resting level by 1.1 L./min./M.^{2} or more, for an average increase of 1.5 L./min./M.^{2} The 5 patients who had improved from Class III to Class II were able to increase their index by an average of 0.9 L./min./M.^{2}

Stroke Index.—In healthy subjects, under the conditions of exercise in this laboratory, cardiac output is increased primarily through an increase in heart

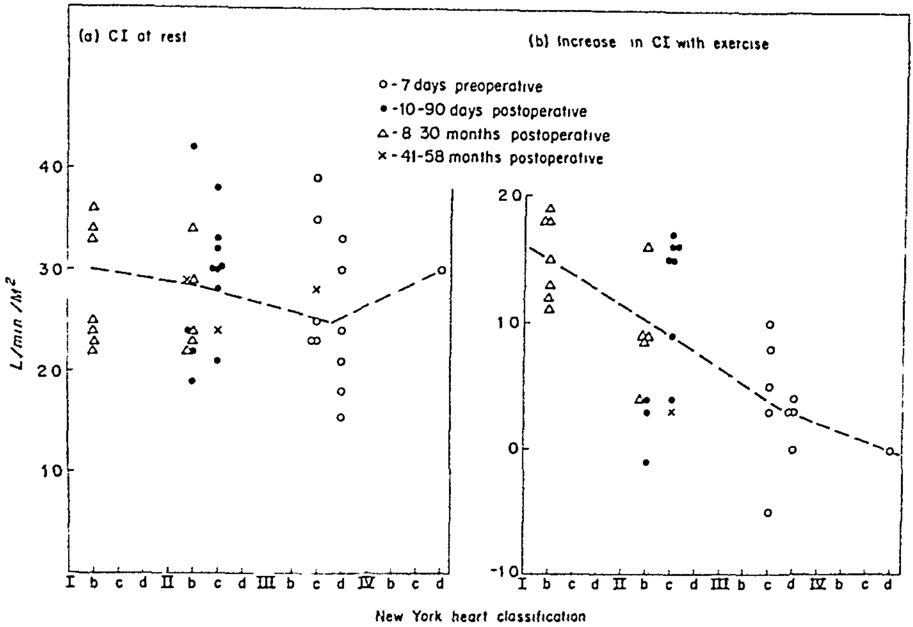


Fig 6.—Relationship of cardiac output at rest (left panel) and its change with exercise (right panel) to the clinical status of the patients before and after mitral commissurotomy The broken line shows the trend of average values. Between the cardiac output at rest and the clinical status of the patient, no correlation is apparent However, the correlation of clinical status with the increase in the cardiac output associated with exercise is significant

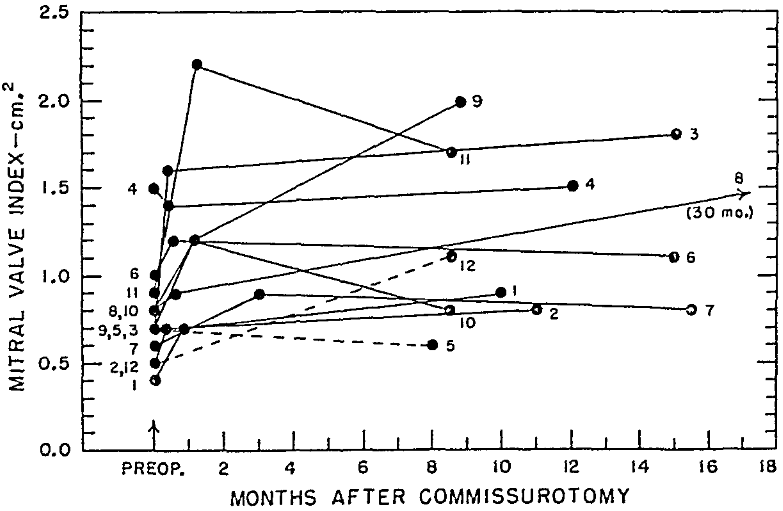


Fig 7.—Mitral valve area index at rest, before mitral commissurotomy and in follow-up studies. Although there was no improvement with respect to this variable in several cases, neither was there significant deterioration during the rest of the period shown (Details are explained in the legend for Fig 1)

rate and the change noted in the stroke index varies between limits of –16.7 to +14.7 ml./beat/min./M.² Only one patient of the present series showed a change in the stroke index outside this range of values preoperatively or postoperatively.

Mitral Valve Area Index.—The formula of Gorlin and Gorlin[1] was used in calculating the mitral valve area index. The theoretical limitations of the formula with reference to actual anatomic conditions are well known, however, it is of value as an index for comparison from patient to patient and particularly for comparison before and after operation in the same patient. In the series of 12 this index averaged 0.8 cm.² preoperatively, 1.2 cm.² 10 to 90 days postoperatively, and 1.2 cm.² 8 to 30 months postoperatively (Fig. 7). Dexter[13] has stated that finger fracture may be expected to increase the mitral valve area by only 30 to 50 per cent. Although in this series the knife was used regularly to supplement finger fracture, the average increase in mitral valve area produced by commissurotomy was similar to that which Dexter observed. But results were not uniform: in 7 cases the calculated postoperative mitral area was twice the preoperative value, and in 5 cases it remained essentially unchanged (Table I).

Blood Pressure and Systemic Resistance.—In the 12 patients of this series the systemic blood pressure tended to be subnormal before commissurotomy, and the total systemic resistance* was consistently high. Normal values for this laboratory according to the data of Barratt-Boyes and Wood[1] are: systemic pressure, 135 systolic and 71 diastolic (standard deviations 15.7 and 7.4) with a mean of 91 (S.D. 8.7), and total systemic resistance, 1,130 ± 178 dyne sec. cm.$^{-5}$

Preoperatively in this series of patients the pressures were: systolic 114 (range 93 to 139), diastolic 66 (range 58 to 86), and mean 82 (range 71 to 97). The average systemic pressures 10 to 90 days postoperatively were: systolic 124 (range 87 to 156), diastolic 65 (range 49 to 83), and mean 89 (range 62 to 104). These figures were practically unchanged at follow-up 8 to 30 months after operation.

The total systemic resistance averaged 1,580 dyne sec. cm.$^{-5}$ (range 965 to 2,470) preoperatively, 1,460 (range 940 to 2,540) at the early postoperative study, and 1,580 (range 1,060 to 2,160) 8 to 30 months postoperatively.

Response to Breathing High Oxygen Mixtures.—In the 12 patients the administration of 99.5 per cent oxygen for 6 minutes or more was followed by reduction in cardiac output in 18 of 31 comparative determinations. The average amount of these 18 reductions was 0.52 L./min./M.² and the average of 8 rises was 0.26, 5 comparisons having been equal. The average change for all 31 was a fall of 0.24 L./min./M.² Studies on normal subjects in this laboratory have shown that the average fall in the cardiac index when breathing high oxygen mixtures is approximately 0.07 L./min./M.², which is not statistically significant.[1]

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*Total systemic resistance was calculated by subtracting the mean right atrial pressure from the mean systemic arterial pressure.

The breathing of 99.5 per cent oxygen was associated with a fall in pulmonary artery mean pressure in 29 of 35 determinations. The average of the 35 changes was a fall of 6 mm. Hg and the average of the 29 falls was 8 mm. Hg.

Statistical analysis of the reduction in cardiac output and the fall in pulmonary artery mean pressure which resulted from breathing 99.5 per cent oxygen in this study reveals that these changes are of a high order of significance. At the present time the explanation for the reduction in cardiac output and pulmonary artery mean pressure is not entirely clear. Systemic resistance apparently rises slightly in response to high oxygen mixtures,[19] and the fall in pulmonary artery pressure is perhaps due to a reduction in pulmonary arteriolar resistance.

In these 12 patients the average change in the right ventricular work against pressure during the administration of 99.5 per cent oxygen, a reduction of 1.5 per cent, is small. However, it is of some clinical significance that in mitral disease, at least, this change in conjunction with the marked increase in oxygen tension in the arterial blood and the increase of approximately 2 volumes per cent in oxygen content may be expected to produce a significant improvement in the transport of oxygen to the body without increasing the work load on the myocardium.

COMMENT

The hemodynamic data available on these patients confirm the clinical impression that most of the improvement to be expected after mitral commissurotomy is likely to occur in the early postoperative period, within perhaps 3 to 4 weeks after operation. Further clinical recovery in the long-term postoperative period has been the experience of many, and the data from this study are consistent with this observation. However, the small gains noted between the early postoperative catheterizations and the recatheterizations performed 8 to 16 months postoperatively may be considered to fall within the limits of accuracy of the techniques employed. What is perhaps most important, the objective data obtained 8 to 30 months after operation indicate that the improvements which mitral commissurotomy produces in various hemodynamic variables are sustained through the long-term recovery period.

Value of Early Postoperative Measurements.—In a consideration of the possibility of recurrent stenosis, data from the early postoperative period are necessary for establishing on an objective basis the degree of relief actually produced by the operation. On the basis of objective data obtained before commissurotomy, soon after it, and in the long-term follow-up as well, it is possible to bring into proper perspective the emotional aspects of this therapy. Without question the psychologic impact of commissurotomy is great in many cases and is, of course, most influential in the early recovery period. The many hemodynamic studies from the early recovery period demonstrate that objective improvement, which usually is slight to moderate, is nevertheless real. In addition, restudy of the 12 patients in this series in the long-term recovery period shows clearly that the hemodynamic gains recorded immediately after mitral commissurotomy remained relatively unchanged in the remainder of the period, 8 to 15 months following operation.

Dexter[18] has speculated on the probable lasting effects of this operation and has suggested that stenosis would not recur except with a recurrence of carditis. Werko and co-workers[1] have stated that without information on the permanence of valvotomy, Group II cases should not be offered this operation —that considerations largely prophylactic do not justify commissurotomy. The data obtained in this study provide some answer to these problems, for it can be said certainly for the group, and with remarkable consistency for the individuals, that the hemodynamic improvement measured at the early postoperative study was maintained for a year or more following operation. These findings are consistent with the observation of Sancetta and co-workers,[9] who catheterized 11 patients before mitral commissurotomy and 6 weeks and 12 months after it, and found that in 7 patients without associated valvular lesions the hemodynamic improvements noted at 6 weeks were maintained to the time of the 12 month study.

Correlation of Objective Data With Clinical Status.—Pulmonary artery mean pressure has been thought the most reliable objective criterion of the severity and symptomatology of mitral stenosis, and many postoperative hemodynamic studies have been based on determinations of this factor and the wedge pressure only. However, Ellis and associates[4] found only poor correlation of pulmonary artery pressure with the degree of disability in cases without operation, and Janton and co-workers[20] noted only very general correlation between the fall in pulmonary artery pressure after commissurotomy and the degree of clinical improvement. Eliasch[21] found the average mean pulmonary artery pressure to be lowest in New York Heart Association Groups I and II, higher in Group III, and highest in Group IV; however, he also noted marked inconsistency in individual cases.

In this study the several physiologic variables determined before and after the operation have been compared with the New York Heart classification groupings of the patients (Figs. 6 and 8). Significant correlations were found between the clinical status of the patient and the pulmonary artery mean pressure, pulmonary artery mean wedge pressure, total pulmonary resistance, pulmonary arteriolar resistance, pulmonary venous resistance, mitral valve area index, and increase in cardiac index with exercise. No correlation was demonstrable between the clinical status of the patient and the resting cardiac index.

Perhaps the hemodynamic variable which usually correlates best with the general clinical status of the individual patient is the magnitude of the increase in cardiac output in response to effort. In this small series (33 comparative determinations), all increases of cardiac index recorded from patients while in Group I were of 1.1 L./min./$M.^2$ or more (average 1.5), while no increase in the patients in Groups III and IV was greater than 1.0 L./min./$M.^2$ (average 0.3). Patients in Group I, then, were able to increase their cardiac output in the low normal range; and patients in Groups III and IV were not. As expected, patients in Group II occupied an intermediate position with respect to this variable and showed an average increase in cardiac index of 0.9 L./min./M.^{2} with several determinations low in the normal range and several well below it.

Although the level of the mean pulmonary artery pressure also shows good general correlation with the clinical classification of these patients, the variations in individual cases point out the desirability of obtaining both pressure and flow data in the evaluation of a given patient.

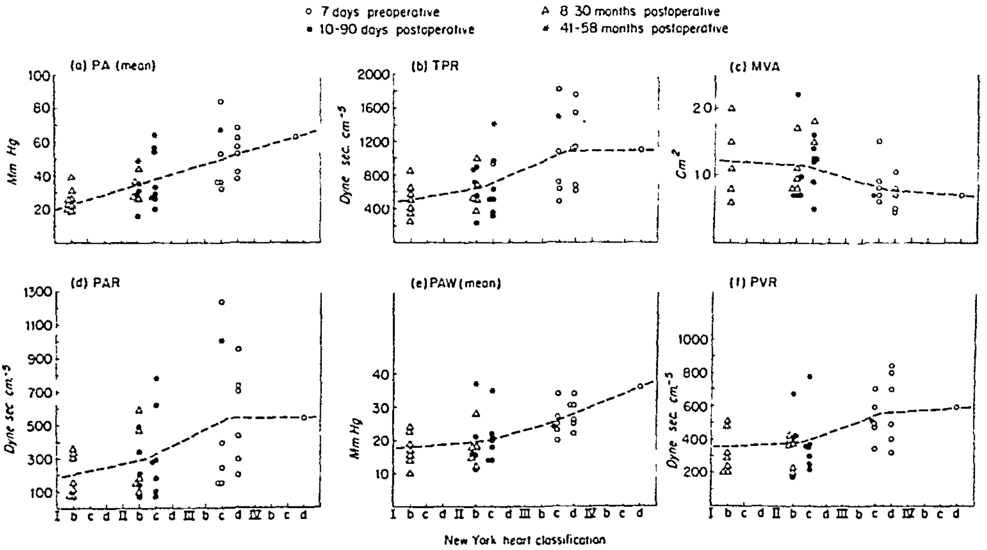

Fig. 8.—Relationship of hemodynamic parameters to the clinical status of the patients before and after commissurotomy. *a*, PA = pulmonary artery mean pressure. *b*, TPR = total pulmonary vascular resistance. *c*, MVA = calculated mitral valve area index. *d*, PAR = pulmonary arteriolar resistance. *e*, PAW = pulmonary artery mean wedge pressure. *f*, PVR = pulmonary venous resistance (TPR-PAR = PVR). The broken line represents the trend of average values within the 4 major classification groups. The correlation between these objectively determined hemodynamic variables and the clinical status of the patient is rough but apparently significant.

The direct effect of commissurotomy on the abnormal hemodynamic values present in mitral stenosis can be appreciated best by noting the degree of the reversion of these values toward normal in individual cases. Reference to Fig. 9, *a*, *b*, and *c* shows that in the present series of patients the postoperative decreases in the pulmonary artery wedge pressure, pulmonary artery mean pressure, and pulmonary arteriolar resistance are directly proportional to the degree of abnormality of these variables before operation. Thus, those patients with the highest pulmonary artery mean and wedge pressures and highest pulmonary arteriolar resistance before operation received the greatest absolute improvement with respect to these variables. Actually, these findings were unexpected, especially with regard to the pulmonary arteriolar resistance. High pulmonary arteriolar resistance has been thought by some to be a relative contraindication to commissurotomy, but the data from this series indicate that the greatest improvement in this variable may be expected in those cases with the most abnormal preoperative values.

Basis of Postoperative Change in Pulmonary Vascular Resistance.—The response of the pulmonary arteriolar resistance to mitral commissurotomy is of considerable theoretical interest. Other studies from this laboratory[22] in which left atrial and pulmonary artery pressures were taken in the operating room immediately before and after opening of the valve have demonstrated that the observed reduction in pulmonary artery mean pressure was closely similar in magnitude to the simultaneous reduction in left atrial pressure achieved by the commissurotomy. Although Fick determinations of cardiac output were not made simultaneously with the pressure recordings, the pressure pulse tracings indicate that cardiac output remained essentially the same for the two sets of pressure determinations. Thus the immediate reduction in pulmonary artery mean pressure as a result of valvotomy appears to be a simple back pressure phenomenon.

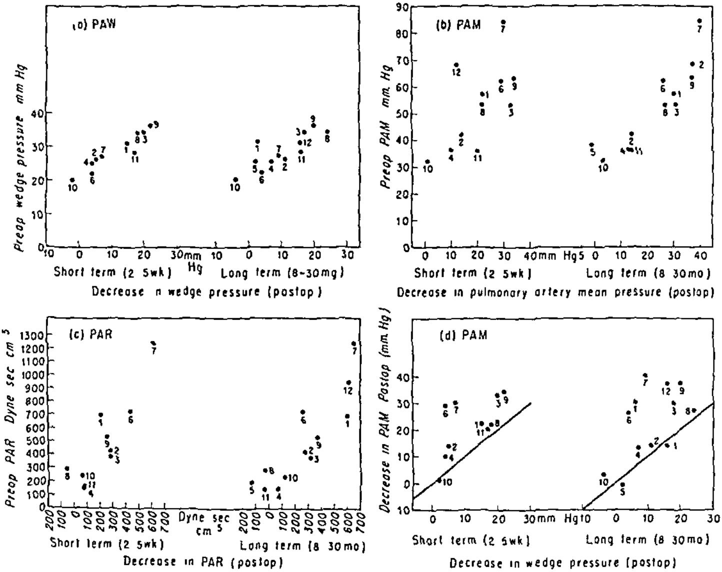

Fig 9.—The relationship of preoperative values of pulmonary artery wedge pressure (*a*), pulmonary artery mean pressure (*b*), and pulmonary arteriolar resistance (*c*) to the decrease in these parameters following mitral commissurotomy. The decrease was directly proportional to their preoperative values. *d*, The relation of the decrease in pulmonary artery mean pressure to the decrease in pulmonary artery wedge pressure following mitral commissurotomy. If the decreases of each kind were equal all points would fall on the diagonal lines. Since the decrease in pulmonary artery pressure exceeds that in the wedge pressure, more than a simple back pressure relationship is involved in this effect. (The numerals indicate the individual patients listed in Table I.)

A further reduction in pulmonary artery resistance, larger proportionately than the reduction in the wedge pressure (Fig 9, *d*), was measured 10 to 90 days after commissurotomy and was found essentially unchanged on recatheterization 8 to 30 months postoperatively. Donald and co-workers[8] also observed that the reduction in the pulmonary artery pressure was greater than the reduction in the wedge pressure 2 years after mitral commissurotomy. It appears, therefore, that the high pulmonary arteriolar resistance in mital stenosis is due at least in part to vasoconstriction, and also that it is partially reversible.

Comparison With Postoperative Effects Reported by Others.—Donald and co-workers[8] have reported recently the hemodynamic findings in 28 patients before successful mitral commissurotomy and 20 months after it. They found that the most consistent hemodynamic effect of the operation was a reduction in the resting cardiac index and a substantial reduction in the pressure work of the right ventricle. The data from the present study also show the reduction in pressure work. However, with regard to the resting cardiac index no consistent fall was found. On the contrary, the majority of patients in this series showed a rise in the resting cardiac index after operation, the rise tending most strongly to occur in those patients whose resting index was subnormal before operation (Table I). With regard to the several other variables determined in studies of this type, the data from the present investigation show a significant correlation with the clinical status of the patient as defined by the New York Heart classification (Figs. 6 and 8). In the study by Donald and associates[8] this relationship was less apparent, perhaps because a number of patients with known combined valvular lesions were included.

Recurrence of Mitral Stenosis.—The problem of recurrent mitral stenosis has been reviewed recently by Belcher.[23] He found opinions varying from a categorical denial of its occurrence to the belief that it occurred only after a recrudescence of carditis or after inadequate valvotomy. He reported 4 cases of clinical recurrence of stenosis in which these factors were thought to have been excluded. Objective hemodynamic evidence demonstrating, first, the improvement effected by successful operation and, second, the regression to the preoperative status, is scarce. Three patients (Cases 6, 9, and 12) in this series presented with clinical evidence of recurrence of stenosis in the third to fifth years following commissurotomy. All 3 had been catheterized 1 week preoperatively and recatheterized 16 to 35 days and 8 to 15 months postoperatively. The recurrence of stenosis was confirmed when they were again recatheterized 41 months to 58 months postoperatively. The 4 sets of data (Table I) demonstrate in an objective way the hemodynamic improvement effected by commissurotomy, the maintenance of this improvement for at least 8 to 15 months postoperatively, and the subsequent recurrence of stenosis in the fourth to fifth years following operation. As mentioned, 2 of these patients have been re-explored and found to have severe mitral stenosis. The third patient has not undergone re-exploration as yet.

Accuracy of Hemodynamic Measurements.—The relatively small degree of change between the immediate and later postoperative values determined by cardiac catheterization is within the suspected margin of error of these measurements in most instances This degree of reproducibility suggests that the actual error in the measurements was small On the other hand, the relatively small change produced by commissurotomy in several of the hemodynamic variables indicates that those parameters must be determined with considerable accuracy in order to demonstrate consistently the objective improvement produced by successful operation The need for both pressure and flow data in the preoperative and postoperative hemodynamic evaluation of these patients is re emphasized

SUMMARY

Hemodynamic data are reported from 12 patients having had right heart catheterization 1 week before, 10 to 90 days after, and 8 to 30 months after satisfactory mitral commissurotomy The data demonstrate that the initial clinical and objective improvement following commissurotomy was sustained for at least 8 to 30 months in each of these patients Although every patient in this series obtained definite clinical and objective hemodynamic benefit following mitral commissurotomy, each had some degree of residual stenosis after the operation All cases have been followed up All the patients are living 4½ to 6 years after operation, and the clinical improvement has been maintained by 9

However, evidence both clinical and objective (from recatheterization and operation) of the recurrence of the preoperative stenotic condition was found in 3 of the cases 3 to 5 years after commissurotomy Recurrence of stenosis cannot be clearly proved without cardiac catheterization soon after operation to establish on an objective basis the actual degree of relief of stenosis obtained through the operative procedure Both accuracy and completeness are essential in these measurements When the measured and derived data from determinations of pressure and of flow are compared with the clinical status of the 12 patients of this series before and after operation, significant correlation is apparent

This study was made possible by the active participation of many clinical and laboratory colleagues The assistance of Drs B Barratt Boyes and H J C Swan, Mrs Jean Frank, and Miss Lucille Cronin was of particular importance

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This product, which was slightly yellow when in solution, was analyzed in the Spinco Model E ultracentrifuge and by paper electrophoresis. In the ultracentrifuge, it sedimented very similarly to another preparation prepared in this institute by phosphate precipitation,[4] i.e., the bulk of the protein sedimented as a tall sharp peak with a sedimentation constant of about 17S; two minor components which sedimented slower and faster than the main component were also present. On paper electrophoresis (phosphate buffer, pH 7.6; $\Gamma/2=0.1$) it migrated with a mobility between that of alpha-1 and alpha-2 globulin. The yellow color of this thyroglobulin preparation remained after two subsequent precipitations in 1.75 M phosphate. There was no apparent antigenic alteration since the final product reacted in an identical way as the thyroglobulin of the original thyroid extract; the purified product was a good antigen in the rabbit.[5]

Ultracentrifugal Fractionation of Hashimoto's Sera.—Hashimoto's sera I, II, and IV were subjected to ultracentrifugation in a partition cell at 59,780 r.p.m., as described previously.[6] The run was terminated after the 19S peak had passed through the partition. The contents of both the upper and lower compartments were analyzed for the presence of autoprecipitins.

THEORY

Human gamma globulin is composed of at least two types of molecules: one with a molecular weight of 160,000, the other with a molecular weight of 1,000,000. These two proteins have a few antigenic groupings in common[3] but are otherwise immunologically distinct and can be identified by the double gel diffusion method even when both are present.[3] The reagent, antiserum against gamma globulin, is easily produced in rabbits, and the two antigens can be easily identified by the characteristic curvatures of the precipitin lines.[8]

Let us assume that the autoprecipitins against thyroglobulin are gamma globulins of either the 7S or 19S variety (or both). Consequently, we would have a system in which these proteins would react with a rabbit antigamma globulin serum as well as with a human thyroglobulin. Let us therefore design the following experiment: in one reservoir of an Ouchterlony plate we place the rabbit antigamma globulin serum, and in the other two reservoirs we place thyroglobulin and Hashimoto's serum, respectively. The patterns of the precipitin lines, depending on whether the 7S or 19S globulin is the autoprecipitin, are illustrated in Diagram 1, *a* and *b*. In both instances it is assumed that only a fraction of either the 7S or 19S globulin is capable of reacting with thyroglobulin. If the autoprecipitin is a 7S globulin, the line formed by it and the thyroglobulin will cross the line formed by the 19S globulin and continue until it reaches the line formed by 7S globulin and the antigamma globulin serum. Since both the thyroglobulin and the antigamma globulin react with the same molecular species, 7S globulin, the two lines will coalesce. If all 7S globulins were antithyroglobulin, the lines would coalesce completely; however, since most of the 7S globulin is inert toward thyroglobulin, it will react with antigamma globulin serum only and a spur will be formed at the site of fusion for similar reasons as described previously,[9] i.e., the inert 7S globulin will diffuse through the precipitate formed by the reactive 7S and the thyroglobulin; once beyond that precipitate the inert 7S will be precipitated by the anti-7S of the rabbit antiserum (Diagram 1, *a*). However, if the antithyroid activity resided in the

19S globulins, then the line formed by Hashimoto's serum and the thyroglobulin would be deflected by the line formed between 19S and the rabbit antiserum (Diagram 1, *b*)

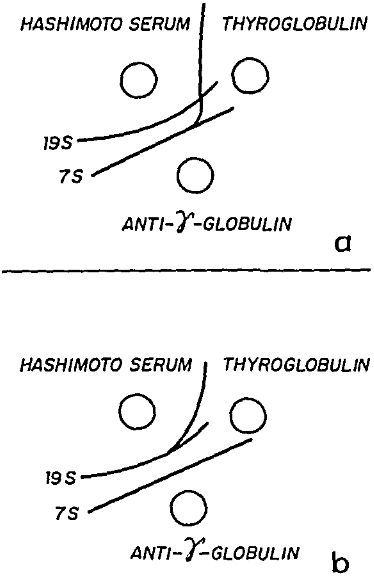

Diagram 1 — The theoretical precipitin patterns to be expected with Hashimoto's sera containing either (a) 7S or (b) 19S precipitins and reacting with thyroglobulin and antibodies against 7S and 19S gamma globulins

RESULTS AND DISCUSSIONS

All the sera studied differed in their immunologic properties toward thyroglobulin (Fig 1) Serum I was extremely potent and still produced one strong precipitin line at a dilution of 1 12 Serum II (Cup D) produced two lines That this was not an artifact is apparent from the following evidence (1) The two lines were produced in the region of equivalence, i e, in the region in which the precipitate remains stationary The production of multiple zones of precipitate with homogenous systems occurs only in the regions of antigen or antibody excess[10] (2) Serum III (Cup C) produced one line only which coalesced with the line closest to the thyroglobulin reservoir (Fig 1) The antigens responsible for the two lines formed with serum II seem to be antigenically related because both coalesced with the single line formed by serum I (Cup B), the resulting pattern is identical to the one previously described for macroglobulins by Korngold and Van Leeuwen,[11] (Diagram 1) and can probably be

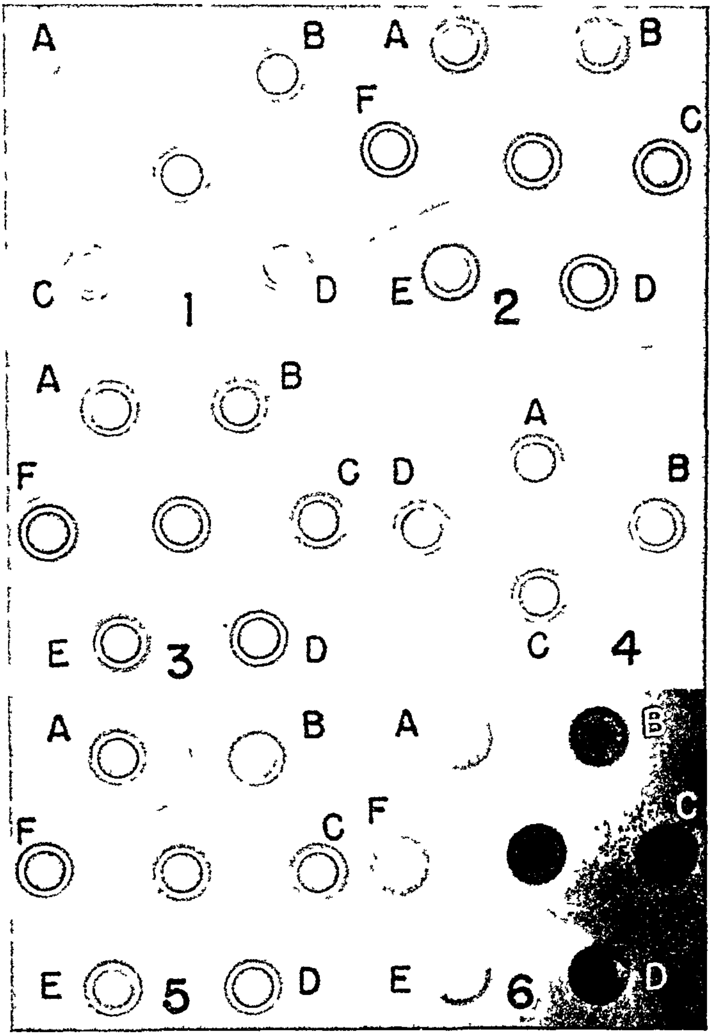

Fig. 1.—Center = Thyroglobulin (7.5 mg./ml.). A = Serum IV. B = Serum I (1:6). C = Serum III. D = Serum II.

Fig. 2.—Center = Thyroglobulin (5 mg./ml.). A = Serum II. B = Anti-γ-globulin serum. C and D = Empty. E = Anti-γ-globulin serum. F = Serum I (1:6).

Fig. 3.—Center = Thyroglobulin (5 mg./ml.). A = Serum II. B = Anti-γ-globulin serum absorbed with 7S gamma-2 globulin. C and D = Empty. E = Anti-γ-globulin serum absorbed with 7S gamma-2 globulin. F = Serum I (1:4).

Fig. 4.—A = Thyroglobulin (5 mg./ml.). B = Serum III. C = Anti-γ-globulin serum absorbed with 7S gamma-2 globulin. D = Serum IV.

Fig. 5.—Center = Thyroglobulin (5 mg./ml.). A = Serum I, top compartment of partition cell (1:3). B = anti γ-globulin serum. C and D = Empty. E = Anti-γ-globulin serum. F = Serum I, bottom compartment of partition cell (1:3).

Fig. 6.—Center = Thyroglobulin (5 mg./ml.). A = Serum IV, bottom compartment of partition cell (1:2). B = Anti-γ-globulin serum absorbed with 7S gamma-2 globulin. C and D = Empty. E = anti-γ-globulin serum. F = Serum IV, top compartment of partition cell. (This plate was treated with Congo red in order to sharpen the contrast between the precipitin lines and the background.)

interpreted in the same manner, i.e., thyroglobulin contains two antigenically related but not identical components, serum I contains antibody against antigenic groupings they both have in common, serum II contains antibody against both common and specific groups, whereas serum III contains antibody specific for one of the components only. That sera from different patients may give rise to either one or two lines has been reported by others,[7, 12] and the pattern produced by serum II is identical to that published by Doniach and associates in their classic paper.[17] Serum IV (Fig. 1, Cup A) also produced one line that coalesced with the lines produced by the other sera. The 4 sera also differed in their potency in that serum I, even at a dilution of 1:6, produced a line that was visible within 24 hours. The lines produced by sera II and III took 2 to 3 days to appear and the sera lost all reactivity if diluted more than 1:4, whereas serum IV could not be diluted more than twofold.

Pressman and co-workers used the late appearances of the precipitin line, as compared with an egg albumin-rabbit anti-EA system, as additional evidence that the precipitin of their Hashimoto's serum was a macroglobulin. However, it should be noted that the time at which the line appears depends on several factors: the strength of the antiserum, the diffusion coefficients of both antibody and antigen, and the concentration of the latter. Since thyroglobulin is a large molecule with a small diffusion coefficient the line would appear later with a 7S antibody than would the line formed with egg albumin, which is a small molecule with a high diffusion coefficient, even if both reactions involved equal amounts of antibody.

The curvature of the lines obtained with sera I, II, and III was toward the antigen (thyroglobulin) reservoir, which suggests that the antigen diffuses more slowly than the antibody.[8] If we could be sure which of the 3 components with different sedimentation constants were the reactive antigens of thyroglobulin, an estimate of the antibody's molecular weight could be made. For example, if the 19S thyroglobulin were the reactive antigen, then the antibody was probably the 7S globulin. However, if the component greater than 19S were the precipitin, the antibody could be either 7S or 19S. (As will be shown later, the component with a sedimentation constant less than 19S is gamma globulin.)

The precipitin line formed by serum IV and thyroglobulin curved away from the antigen reservoir (Figs. 1 and 4), suggesting that the antibody diffused more slowly than did the thyroglobulin.[8] The diffusion coefficients of gamma 1 macroglobulin and thyroglobulin are approximately 1.8 and 2.6, respectively,[1] which agrees with the observed curvature of the precipitin lines. The differences in curvature of the lines produced by sera from patients with Hashimoto's struma is illustrated in Figs. 1 and 4. In this study, where the physical characteristics of the antigen are known, it is possible to obtain information about the nature of the antibodies from the curvature of the precipitin lines. However, for other less well defined systems it will be essential to employ the method described above. The results with this immunologic method strongly indicate that the autoprecipitins of sera I, II, and III are 7S gamma globulins

For example, from Figs. 2 and 3 it can be seen that the lines formed by thyroglobulin and Hashimoto's sera I, II, and III intersect the line formed by the 19S gamma globulin of Hashimoto's serum with antigamma globulin. This lack of interference between the two lines is even more apparent in Fig. 3 where the antigamma globulin serum was adsorbed with 7S gamma globulin. The pattern of the lines in Figs. 2, 3, and 5 resembles that of Diagram 1, *a,* and consequently one may conclude that the thyroglobulin reacts with 7S autoprecipitin rather than with 19S globulin. The use of undiluted Hashimoto's sera (Fig. 2, Cup A; Fig. 6, Cup F) resulted in very broad zones of precipitate owing to the high concentration of 7S gamma globulin, this being in extreme antigen excess relative to the antigamma globulin serum. Such zones have been described by others.[13]

The fact that the unadsorbed antigamma globulin serum also reacts with a component of the thyroglobulin preparation (Figs. 2 and 5) and that the resulting line coalesces with the line formed by the 7S gamma globulin of Hashimoto's serum indicates that the thyroglobulin is still contaminated with some gamma globulin as was pointed out above. It has been found by others[14] that molecules immunologically indistinguishable from 7S gamma globulin can be found in the alpha-2 globulin fraction; however, it is of some interest that we have been unable to detect this component in alpha-2 globulin isolated from serum by zone electrophoresis on starch, even in concentrations of 10 mg. per milliliter. From Fig. 4 it can be seen that the line formed by Hashimoto's serum IV (Cup D) and thyroglobulin (Cup A) never crossed the line formed by the 19S globulin and antigamma globulin serum (Cup C). Since the pattern is the same as pictured in Diagram 1, *b,* it may be concluded that the autoantibody of serum IV is a 19S gamma globulin similar to the one described by Pressman and co-workers.[2] The same plate (Fig. 4) contains for comparison the pattern obtained with a 7S autoprecipitin of serum III (Cup B). It can be seen that the two lines intersect and that the curvature of the line formed by 7S precipitin and thyroglobulin is quite different from that of the 19S precipitin of serum IV (Cup D) and thyroglobulin. In a recent paper, which appeared while this manuscript was in preparation, Roitt and associates,[15] concluded from the curvature of the precipitin line obtained with Hashimoto's serum and thyroglobulin that the antigen was a large, slowly diffusing molecule. This, of course, is based on the assumption that the antibody was a 7S globulin.

For further confirmation of our data, Hashimoto's sera were subjected to ultracentrifugal fractionation in a partition cell, and both the top and bottom compartments were tested for the presence of autoprecipitins and 19S gamma globulins. Hashimoto's serum I fractions were diluted 1:3. It should be pointed out that the bottom fraction will always contain the active component at a higher concentration than that of the original serum since the bottom compartment was filled with the original serum. From Fig. 5 it can be seen that the contents of the top compartment (Cup A) produce a very strong line with thyroglobulin (center), although only a faint line, which could not be photographed, was formed between anti-19S globulin and traces of 19S globulin which were still present in the top fraction of serum I It should be realized that the immunologic test for 19S is very sensitive, this particular antiserum can detect it even when only 5 per cent of the original macroglobulin of the serum is present

The results obtained with serum II were similar to those described for serum I

The ultracentrifugal results obtained with serum IV were in agreement with the immunologic data, i e , hardly any activity was found in the undiluted contents of the top compartment of the partition cell (Fig 6, Cup F) whereas the contents of the bottom compartment (Cup A) reacted at a dilution of 1 2

Therefore, from the immunologic and ultracentrifugal data presented, it appears that the autoprecipitins of Hashimoto's sera may be either 7S or 19S gamma globulins Hashimoto's sera I, II, and III contained the 7S antibody, whereas serum IV contained the 19S precipitin The examination of many more sera will ultimately decide whether the 7S or 19S globulin is the more common form of this autoprecipitin

SUMMARY

A new method for the determination of the molecular weight of autoprecipitins is presented This method depends on the antigenic specificity of the 7S and 19S gamma globulins commonly found in normal sera, and their easy identification with proper antisera in the Ouchterlony double gel diffusion technique

The examination of Hashimoto's sera containing autoprecipitins against human thyroglobulin by this method has shown that they are either 7S or 19S globulins, these data were confirmed by ultracentrifugal fractionation of 3 of these sera

Human thyroglobulin is shown to contain two antigenically related components, a third component is antigenically identical with gamma globulin and is probably a contaminant

We wish to thank Drs B Ruebner and R Benua for sera III and IV We are also very much indebted to Dr Mary L Petermann for her advice and the use of the analytic ultracentrifuge as well as to Mrs Mary G Hamilton for fractionating 3 of the sera in the partition cell The able technical assistance of Mr Rudolph Smith is acknowledged

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VITAMIN B_{12} ABSORPTION RELATIONSHIP BETWEEN ORAL ADMINISTRATION AND URINARY EXCRETION OF $COBALT^{60}$ LABELED CYANOCOBALAMIN FOLLOWING A PARENTERAL DOSE

STUDY OF DOSES OF 2 TO 250 µg IN 148 APPARENTLY HEALTHY MEN 20 TO 92 YEARS OLD

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ABSORPTION of orally administered radioactive cobalt labeled vitamin B_{12} (cyanocobalamin or derivative) has been estimated in normal human subjects by difference in the amount of radioactivity administered and that recovered in the feces[1] Absorption has also been correlated with hepatic accumulation[6] and plasma levels[11] following oral administration of labeled vitamin B_{12}, and with radioactivity appearing in the urine after oral administration of the labeled vitamin followed by a large parenteral dose of unlabeled material[11] Correlations between urinary excretion of absorbed vitamin B_{12} and plasma levels[9] and between results from fecal and urinary excretion tests[17] have been demonstrated The reliability of the urinary excretion technique for distinguishing between normal and markedly impaired absorption of vitamin B_{12} for smaller oral doses (from 0 1 to 2 µg, usually about 0 5 µg), has been confirmed in many laboratories,[3, 4, 12, 16] although abnormally low excretions of radioactive vitamin B_{12} have been noted in terminal cardiorenal disease and in severe renal disease[1]

The present study was designed, primarily to evaluate, in healthy subjects, the effect on urinary recovery following a parenteral dose, of varying the oral dose of vitamin B_{12} over a wide range, extending from physiologic or near physiologic levels to several hundred times these levels A second consideration has been exploration for possible age differences in absorption for such doses The dose levels we have selected for study (2 to 250 µg) are higher than those at which an intrinsic factor deficiency, if existent, would seem most likely to be detected, since smaller overlap between results for normal subjects and pernicious anemia patients has been observed when the oral dose was less than 1 µg than when a larger dose was used[4, 13, 18] Thus, for the higher doses of the present study at least, exploration for age differences constitutes study of agewise variation in efficiency of absorption via mechanisms relatively independent of the amount of intrinsic factor available While furnishing information on a

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TABLE I. RENAL FUNCTION STUDIES FOR FOURTEEN ELDERLY SUBJECTS IN PRESENT STUDY (VALUES FROM LITERATURE FOR AGEWISE COMPARISON)

	ELDERLY SUBJECTS OF PRESENT STUDY									HEALTHY MEN STUDIED BY DAVIES AND SHOCK[19]*					
AGE WHEN RENAL FUNCTION STUDIED (YEARS)	NUMBER OF SUBJECTS	INTERVAL BETWEEN RENAL FUNCTION AND PRESENT STUDIES (YEARS)		INULIN CLEARANCE (ML./MIN./1.73 M.^{2})		DIODRAST OR PAH CLEARANCE (ML./MIN./1.73 M.^{2})		Tm DIODRAST OR PAH (MG. I/MIN./1.73 M.^{2})		INULIN CLEARANCE (ML./MIN./1.73 M.^{2})		DIODRAST CLEARANCE (ML./MIN./1.73 M.^{2})		Tm DIODRAST (MG. I/MIN./1.73 M.^{2})	
		MEAN	RANGE	MEAN	RANGE	MEAN	RANGE	MEAN	RANGE	MEAN	S.D.	MEAN	S.D.	MEAN	S.D.
20-29	–									123	16	614	75	54.6	9.5
30-39	–									115	11	649	117	51.0	8.6
40-49	–									121	23	574	112	49.9	9.8
50-59	1	5	–	85.8	–	481†	–	74.7†	–						
		3	–	96.0	–	432	–	60.9	–	99.3	14.6	500	87	45.3	6.3
60-69	1	5	–	81.0	–	342	–	–	–	96.0	25.5	442	80	44.5	9.1
70-79	7	3	1-7	76.6	60.3–90.0	313†	174–427†	66.3†	46.3–75.6						
	1	4	–	69.3	–	242	–	39.2	–	89.0	19.9	354	83	39.1	7.3
80-89	3	3	2-4	55.7	38.6–66.1	266†	155–358†	52.4†	33.5–65.3†	65.3	20.4	289	89	30.8	9.8

*Reported values are rounded off in some cases.

†Indicates PAH (para-aminohippurate).

wide range of doses for which data are meager, our results are also of interest because they represent a large group of healthy subjects studied under conditions in which complete urine collections were facilitated by continuous observation of the subjects during the period of the collections.

MATERIALS AND METHODS

Subjects were 148 men selected from the Gerontology Ward and Chronic Division of the Baltimore City Hospitals (55 to 92 years old) and from a penal institution in the same geographical area (20 to 39 years old). Subjects from the Gerontology Ward and Chronic Division had been there for several years, primarily for indications which were socioeconomic and associated with advanced age rather than illness. Subjects were selected, after review of all available clinical information, as those who were in good general health. All were ambulatory, free of acute or debilitating chronic illness, were in apparently good nutritional status, and believed to be free of hematologic or gastrointestinal disorder. In no subject was there reason for suspicion of diabetes mellitus Except for the requirement of urinary continence and the general criteria noted above, there was neither an attempt to select subjects with respect to renal function, nor any clinical indication for suspicion of abnormal function. Status of renal function has been evaluated in 14 of the older subjects as a part of other investigations of the Gerontology Branch on renal function in healthy middle aged and elderly men Renal function measurements for these subjects* are given in Table I and are seen to be fairly typical for their age groups when compared with the group of healthy subjects studied by Davies and Shock[19] There was no reason to believe, after review of all available information, that any of the subjects had been administered a source of vitamin B_{12}, except as contained in the institutional diets[20] during the preceding year

Each subject was given orally (in aqueous solution followed by 2 rinses) 2, 8, 50, or 250 μg of Co^{60} labeled vitamin B_{12}† (about 2 hours after breakfast and 2 hours before lunch) followed 2 hours after the ingestion of the radioactive vitamin by the intramuscular injection of 1,000 μg of nonradioactive vitamin B_{12}[11] Following the ingestion of the labeled material, urine was collected for either (1) 0 to 12, 12 to 24, and 24 to 48 hours, or (2) 0 to 24 hours only. When collections at 0 to 12 and 12 to 24 hours were made, results were added to obtain a value for 0 to 24 hour excretion of radioactive vitamin To facilitate complete urine collection, all subjects were under continuous observation during the collection periods The procedure used for measurement of the radioactivity excreted into the urine was as previously reported[21] except that the aliquot used for measurement in the present studies was half of that collected That the excreted radioactivity represents "tagged" vitamin B_{12} has been indicated by chromatographic and other analytic studies,[22] and by differences in storage of radioactivity after $Co^{60}Cl_2$ and Co^{60} vitamin B_{12} administration[23]

RESULTS

The results, summarized in Fig 1, show the effect on the per cent of orally administered radioactive vitamin B_{12} recovered in the urine, of varying (1) the oral dose of $Co^{60}B_{12}$ in amounts of 2, 8, 50, and 250 μg (Fig 1, *A*, *B*, *C*, and *D*, respectively) and (2) the urine collection intervals (12, 24, and 48 hours) for each of the age groups and the total group.

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*We are indebted to Drs Nathan W Shock and David Oursler, of the Gerontology Branch, for making available to us the renal function data on subjects included in the present study

†For supplies of radioactive and nonradioactive vitamin B_{12} we are indebted to Dr Charles Rosenblum and Merck & Company Inc The present studies were authorized by the Atomic Energy Commission (Authorization Nos 34126 and 22808), and we gratefully acknowledge the partial support given in a grant-in-aid from the AEC under contract No. AT(30-1) 1203

Variations With Age.—The data given in Fig. 1 showed no systematic age difference, for any of the doses or collection periods, in the fraction of dose of cobalt^{60}-vitamin B_{12} excreted in the urine under the experimental conditions of the present study. Further statistical evaluation, in particular for the 24-hour collections when the group aged 20 to 39 years was compared with the group aged 55 to 92 years, revealed no significant age difference: 2 μg, $P > 0.2$; 8 μg, $P > 0.1$; 50 μg, $P > 0.05$; 250 μg, $P > 0.7$. Even when (for 24-hour collections) the group 20 to 39 years old was compared with that 75 years old and older, no consistent age trend was found: 2 μg, $P > 0.1$; 8 μg, $P > 0.01$; 50 μg, $P > 0.6$; 250 μg, $P > 0.2$.

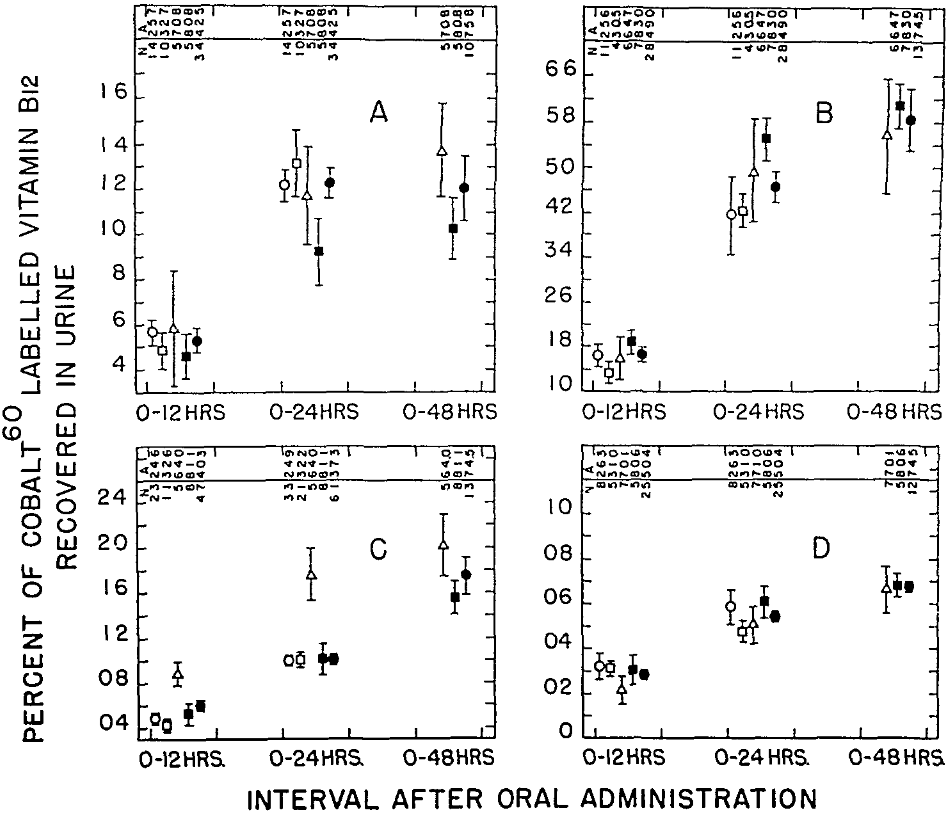

Fig. 1.—Effect, on the per cent of orally administered radioactive vitamin B_{12} recovered in the urine, of varying the oral dose for doses of 2, 8, 50, and 250 μg (A, B, C, and D, respectively), and the urine collection interval (12, 24, and 48 hours) for each of the age subgroups and the combined group of healthy men. Key to symbols: ○ = mean Co^{60}-vitamin B_{12} recovery for subjects of ages 20 to 29 years; □ = mean Co^{60}-vitamin B_{12} recovery for subjects of ages 30 to 39 years; △ = mean Co^{60}-vitamin B_{12} recovery for subjects of ages 55 to 74 years; ■ = mean Co^{60}-vitamin B_{12} recovery for subjects of ages 75 to 92 years; ● = mean Co^{60}-vitamin B_{12} recovery for subjects of all ages combined. Vertical lines define limits of the standard error of the mean. At the top of each diagram is given the number of subjects in each age subgroup (N) and the average age for the subjects in the subgroup (A).

Variations With Collection Interval.—For all of the oral doses considered, the amount of vitamin B_{12} recovered in the 12- to 24-hour urine collection was greater than for the 0- to 12-hour collection. Except for the 2 μg dose, the amount of vitamin B_{12} appearing in the collection at 24 to 48 hours was one fifth to one half as much as that recovered in the first 24 hours after oral administration. For the 2 µg dose, only small amounts of vitamin B_{12} were recovered in the 24- to 48-hour urine collection.

Variations With Oral Dose.—It is apparent from Fig. 1 that with an increasing amount of vitamin B_{12} administered orally, there was a marked decrease in the *fraction* recovered in the urine, under, of course, the conditions of this study. As the oral dose was increased, however, the calculated *amount* of vitamin B_{12} recovered in the urine definitely increased, as shown by the mean values and standard deviations in Table II.

DISCUSSION

A. Effect of Parenteral Dose and Tissue Retention on Results.—The urinary excretion of orally administered vitamin B_{12} in the present study represents only a minimum value for the amount absorbed. The observed difference between the amount apparently absorbed and that excreted in the urine[1] is due, to some extent, to the parenteral dose, since this has been observed to decrease the proportion of an oral dose absorbed from the gastrointestinal tract.[1, 24] The effect of the parenteral dose, however, would seem to be small compared to that due to tissue retention, a large proportion of the absorbed material is retained in the liver.[8, 25] From these considerations and the results of the present study, it would appear that the average amount of vitamin B_{12} absorbed from a single 250 µg dose probably greatly exceeds 1.7 µg, since the latter was the average amount of labeled vitamin B_{12} recovered in the urine within 48 hours after the administration of 250 µg of labeled material.

B. Relation of Amount and Time of Vitamin B_{12} Absorption to Oral Dose.—The character of the relationship obtained in the present study, between oral dose and the amount of vitamin B_{12} excreted in the urine, seems to be in remarkably good agreement with that indicated by the results of Glass and associates.[25] These workers, using the hepatic uptake technique and oral dose range of 0.5 to 50 µg, noted increase in amount and decrease in fraction taken up by the liver in subjects given larger oral doses compared to subjects given smaller doses of labeled vitamin B_{12}. While in agreement with this work, our findings differ from the conclusions of Callender and Evans[1] and Swenseid and co-workers,[7] who used the fecal excretion technique and found optimal absorption at doses not exceeding about 2 and 5 µg, respectively. It seems conceivable that the "plateau" in absorption observed by the latter 2 groups was due to interference associated with successive tests in the same subjects.

The distributions of radioactivity between the several urine collections, in the present study, are consistent with the view that vitamin B_{12} absorption proceeds gradually, and approaches maximal levels sooner for smaller oral doses.

The relationship of the fraction and amount of vitamin B_{12} recovered in the urine to the oral dose of the vitamin are consistent with the views that

TABLE II. AMOUNTS OF VITAMIN B_{12} RECOVERED IN THE URINE FOR VARIOUS ORAL DOSES, FOLLOWED BY A PARENTERAL DOSE, IN APPARENTLY HEALTHY MEN OF AGES 20 TO 92 YEARS

ORAL DOSE (μg)	RECOVERIES OF VITAMIN B_{12} IN URINE (MICROGRAMS)											
	0 TO 12 HOURS				0 TO 24 HOURS				0 TO 48 HOURS			
	N*	RANGE	MEAN	S.D.†	N	RANGE	MEAN	S.D	N	RANGE	MEAN	S.D.
2	34	0.002 0.28	0.11	0.06	34	0.09-0.48	0.24	0.08	10	0.11-0.41	0.24	0.09
8	28	0.008 0.23	0.13	0.06	28	0.13-0.70	0.37	0.11	13	0.21 0.80	0.46	0.16
50	47	0.06 0.77	0.25	0.14	61	0.33-1.22	0.55	0.17	13	0.45-1.30	0.84	0.30
250	25	0.30-1.65	0.70	0.20	25	0.63-2.53	1.35	0.24	12	0.68-2.80	1.66	0.21

*N = number of subjects.

†S D = standard deviation of distribution.

there are at least 2 mechanisms of absorption of vitamin B_{12} from the gastrointestinal tract* in normal people, that these mechanisms act concurrently, and that at different oral dose levels the 2 mechanisms are of relatively different *importance*. Thus, *at low (physiologic) dose levels, the available intrinsic factor* is of relatively great importance in influencing the amount absorbed, while at the higher dose levels intrinsic factor is of decreased relative importance and the amount absorbed seems to be more directly related to the amount administered, i.e., a "mass" effect. Although it has not yet been clearly demonstrated *to mediate vitamin B_{12} absorption, diffusion would seem to be the simplest* mechanism consistent with existing observations of absorption apparently beyond that mediated by intrinsic factor, insofar as these data have been analyzed. It would seem reasonable to suppose that absorption by this mechanism would be negligible or, at least, inadequate to meet nutritional requirements, at low *(physiologic) dose levels. At usual dietary levels then, adequate amounts of* the vitamin would be expected to traverse the intestinal mucosa only if adequate amounts of intrinsic factor were available. The manner by which this traversal comes about (at low and high oral dose levels), while the subject of conceptually appealing speculations[illegible] with which our results are not inconsistent, *will remain speculative until many more data have become available and adequately interpreted*.

C. Age Differences.—

1. Effect on results of age differences in renal function. In consideration of the possible effect of renal function on the results, it should be noted that subjects included in the present study probably have renal function rather typical for healthy subjects in their respective age groups (see Introduction and Table I). Even for healthy men, however, there is regression of renal function on age.[19] In the absence of such influence in our older subjects however, the values for excretion of vitamin B_{12} might have been *higher* but not lower than those we observed. Had it happened that significantly less radioactive vitamin B_{12} had been excreted by the older, as compared to the younger, subjects, then the difference might possibly have been explained on the basis of lower renal function in the older subjects. Such did not occur, however, and the fact that the urine collections for the older men contained as much radioactivity as did those for the young subjects cannot be explained on the basis of age differences in renal function.

Failure to observe decreased excretion of Co^{60} vitamin B_{12} in the older subjects we have studied was not likely to have been due to decreased tissue retention of absorbed vitamin B_{12}, in the old as compared to the young men, in view of the following considerations: (1) amounts of radioactive vitamin B_{12} retained by the liver after oral administration were found by Glass and coworkers[5] to be not significantly different in old and young men, (2) the large parenteral dose of vitamin B_{12} given to all subjects, would be expected to

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*Since this paper was submitted there has appeared a paper by Doscherholmen and Hagen which supports the existence of a dual mechanism in the absorption of vitamin B_{12} (Doscherholmen, A., and Hagen, P. S.: A Dual Mechanism of Vitamin B_{12} Plasma Absorption. J. Clin. Invest. 36: 1551-1557, 1957.)

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A SEROLOGIC AND ELECTROPHORETIC STUDY OF THE MALIGNANT AND PROLIFERATIVE DISORDERS OF THE HEMATOPOIETIC AND RETICULOENDOTHELIAL SYSTEMS

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The distribution and concentration of various plasma protein constituents are altered in many disorders of the hematopoietic and reticuloendothelial systems. Such changes have been demonstrated by electrophoretic[6] and serologic techniques[7] in patients with lymphoma, leukemia, and multiple myeloma.

The purpose of this paper is to study the serum protein changes in patients with malignant hematopoietic disorders by the use of electrophoretic and serologic techniques and to relate these variations to the type of disease, the clinical status of the patients, and the effect of therapeutic agents employed in the treatment of these disorders.

MATERIALS AND METHODS

The subjects of this study were 99 patients admitted to the hematology clinic or wards of the First and Third Medical Services (Tufts), Boston City Hospital, between September, 1957, and February, 1958, in whom the diagnosis of malignant lymphoma, leukemia, myeloproliferative disorder, or multiple myeloma was confirmed by peripheral blood studies and bone marrow aspiration and/or tissue biopsies. Healthy adults, both men and women, 20 to 45 years of age, served as controls.

Serum protein filter paper electrophoresis was performed according to a modification of the methods of Williams and associates[8] and Jencks and co-workers,[9] using a Spinco Model R duostat or Heathkit duostat as the power source, a Spinco Model R paper electrophoresis cell, Spinco filter paper wicks and strips, and barbital buffer, pH 8.6, ionic strength 0.075, and 0.1 M. Electrophoresis of the sera was performed at 250 volts over a 6 hour period. The strips were dried in an oven at 45° C., stained with bromphenol

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give a higher degree of tissue saturation with the vitamin (before the radioactive vitamin entered the plasma) than would otherwise have been the case; (3) retention of parenterally administered doses of 20 to 75 μg of vitamin B_{12} is probably even greater in old men than in young men.[28]

2. *Comparison of our results with those of other studies related to absorption of vitamin B_{12} and aging:* Chow and associates[21] measured the urinary excretion of radioactive vitamin B_{12} following oral administration of 2 μg of labeled material and a large parenteral dose; in 40 healthy subjects 19 to 85 years old, no significant age difference was observed. Using the hepatic uptake method and a test dose of 0.5 μg of vitamin B_{12}, Glass and co-workers[6] found no significant age difference in radioactive vitamin B_{12} uptake by the liver in 60 subjects aged 18 to 90 years who were normal or had some irrelevant disorder. In other reports, the number of "healthy" or "control" subjects has been inadequate for statistical treatment of the data for agewise study (8 ''controls'' in Schilling's[29] paper, for example) or adequate information on ages of subjects has been omitted.[12]

3. *Relation of the present study to evidence for age differences in serum vitamin B_{12} levels:* Recent studies in healthy human subjects in our laboratories[20] and elsewhere[30, 31] have indicated that there is a decrease in serum concentration of vitamin B_{12} microbiologic activity with advancing age. The results of the present study for doses of 2 to 250 μg, other studies[21] for the 2 μg dose, and studies of hepatic uptake for the 0.5 μg dose[6] do not support the contention that age differences in serum levels of vitamin B_{12} are due to impairment of absorption of the vitamin. It is possible, although not yet clearly demonstrated, that the lower levels of serum vitamin B_{12} observed in the aged subjects may have been associated with decreased ingestion of vitamin B_{12} due to dietary selection by the subjects themselves,[20] in the absence of any significant impairment in vitamin B_{12} absorption. It should be emphasized, however, that tests using oral doses of 2 μg and larger, such as in the present study, are not representative of absorption for the smaller doses in which the best separations have been found between patients with intrinsic factor deficiency and normal subjects.[1, 13, 18] Smaller doses, moreover, would seem to closely approach levels more frequently found in the diet (and are, in this sense, physiologic doses). The data do suggest, however, that amounts of vitamin B_{12} absorbed from oral doses in the 2 to 250 μg range are not significantly less in old men than in young men.

SUMMARY

1. Labeled material appearing in the urine in 12, 24, and 48 hours after oral administration of 2, 8, 50, or 250 μg of cobalt^{60}-labeled vitamin B_{12} (cyanocobalamin or derivative), followed after 2 hours by intramuscular injection of 1,000 μg of nonradioactive vitamin B_{12}, has been estimated in 148 apparently healthy men 20 to 92 years old.

2. With increasing oral dose, for the range considered, there was observed marked decrease in *fraction* but considerable increase in *amount* of vitamin

B_{12} appearing in the urine. For all doses, the amount of vitamin B_{12} recovered in the 12- to 24-hour urine collection was greater than for the collection at 0 to 12 hours. Except for the 12 μg dose, the amount of vitamin B_{12} appearing in the 24- to 48-hour collection was one-fifth to one-half as much as that recovered in the first 24 hours after oral administration. Mean recoveries in micrograms of labeled vitamin B_{12} in the urine ± standard deviation of distribution for each of the oral doses and for 0 to 12, 0 to 24, and 0 to 48 hours after oral administration, respectively, were: (1) *2 μg:* 0.11 ± 0.06, 0.24 ± 0.08, 0.24 ± 0.09, (2) *8 μg:* 0.13 ± 0.06, 0.37 ± 0.11, 0.46 ± 0.16, (3) *50 μg:* 0.25 ± 0.14, 0.55 ± 0.17, 0.84 ± 0.31. (4) *250 μg:* 0.70 ± 0.20, 1.35 ± 0.24, 1.66 ± 0.21.

3. The data are consistent with the views that there are at least two mechanisms of absorption of vitamin B_{12} from the gastrointestinal tract, that these mechanisms act concurrently and have relatively different importance at different dose levels, and that absorption proceeds gradually, approaching maximal levels sooner for smaller dose levels.

4. Age differences in urinary excretion of orally administered material were not demonstrated for any of the doses under the experimental conditions of the study. The oral doses used, however, were higher than those for which best separation has been demonstrated between normal subjects and patients with intrinsic factor deficiency. The excreted material probably represents that absorbed, to some extent for the smallest dose and in large proportion for the larger doses, by a mechanism relatively independent of the amount of intrinsic factor available. The efficiency of such a mechanism, insofar as indicated by the urinary excretion test, was not impaired in the older, as compared to the younger, subjects.

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A SEROLOGIC AND ELECTROPHORETIC STUDY OF THE MALIGNANT AND PROLIFERATIVE DISORDERS OF THE HEMATOPOIETIC AND RETICULOENDOTHELIAL SYSTEMS

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THE distribution and concentration of various plasma protein constituents are altered in many disorders of the hematopoietic and reticuloendothelial systems. Such changes have been demonstrated by electrophoretic[26] and serologic techniques[27] in patients with lymphoma, leukemia, and multiple myeloma.

The purpose of this paper is to study the serum protein changes in patients with malignant hematopoietic disorders by the use of electrophoretic and serologic techniques and to relate these variations to the type of disease, the clinical status of the patients, and the effect of therapeutic agents employed in the treatment of these disorders

MATERIALS AND METHODS

The subjects of this study were 99 patients admitted to the hematology clinic or wards of the First and Third Medical Services (Tufts), Boston City Hospital, between September, 1957, and February, 1958, in whom the diagnosis of malignant lymphoma, leukemia, myeloproliferative disorder, or multiple myeloma was confirmed by peripheral blood studies and bone marrow aspiration and/or tissue biopsies Healthy adults, both men and women, 20 to 45 years of age, served as controls.

Serum protein filter paper electrophoresis was performed according to a modification of the methods of Williams and associates[28] and Jencks and co workers,[29] using a Spinco Model R duostat or Heathkit duostat as the power source, a Spinco Model R paper electrophoresis cell, Spinco filter paper wicks and strips, and barbital buffer, pH 8.6, ionic strength 0.075, and 0.1 M. Electrophoresis of the sera was performed at 250 volts over a 6 hour period. The strips were dried in an oven at 45° C, stained with bromphenol

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From the Tufts Hematology Laboratory, First and Third (Tufts) Medical Services, Boston City Hospital, and The Mallory Institute of Pathology, Boston City Hospital

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TABLE II. SERUM PROTEIN DISTRIBUTION AND CONCENTRATION IN PATIENTS WITH HODGKIN'S DISEASE

	VALUES	NUMBER OF PATIENTS	NUMBER OF OBSERVATIONS	ALBUMIN (%)	ALPHA-1 GLOBULIN (%)	ALPHA-2 GLOBULIN (%)	BETA GLOBULIN (%)	GAMMA GLOBULIN (%)	TOTAL PROTEIN (GM. %)
Group 1 (patients in relapse)	Mean	9	18	39.3	8.1	15.2	16.9	21.6	6.0
	Standard deviation			7.9	2.8	3.4	3.5	6.6	0.9
Group 2 (patients in remission)	Mean	11	27	46.3	7.2	13.7	16.1	16.9	6.4
	Standard deviation			10.4	2.1	4.8	5.4	5.9	1.0
Patients in relapse compared with controls	Significance (p)			0.001↓*	0.007↑*	0.02↑	0.001↑	0.4	0.06
Patients in remission compared with controls	Significance (p)			0.70	0.01 ↑	0.5	0.3	0.008↓	0.1
Patients in relapse compared with patients in remission	Significance (p)			0.01 ↓	0.2	0.2	0.6	0.02 ↑	0.001↓

In this and the following tables, ↓ refers to significantly decreased values and ↑ refers to significantly increased values consisted of cases of Hodgkin's paragranuloma, Hodgkin's granuloma, and Hodgkin's sarcoma. Nine cases in relapse received varying forms of therapy during the course of study, including prednisone (2 cases), nitrogen mustard (2 cases), and x-irradiation and nitrogen mustard (5 cases).

In the sera of patients in relapse, the albumin levels were significantly decreased below normal, while the alpha 1, alpha 2, and beta globulin fractions were significantly elevated. The sera from 11 patients in remission exhibited two significant deviations: an increase in alpha 1 globulin values and a decrease in gamma globulin levels. The albumin and total protein levels of the sera from patients in relapse were significantly lower, and the gamma globulin levels were significantly higher than the corresponding values in the sera from the patients in remission.

The distribution of isoagglutinin titers for the patients with Hodgkin's disease is shown in Fig. 1, A. There was no difference between the patients and controls. There was no correlation between gamma globulin concentration and isoagglutinin titers.

Malignant Lymphoma.—This group consisted of 19 cases of lymphocytoma, lymphoblastoma, lymphosarcoma, and reticulum cell sarcoma. Nine patients were in remission. Ten patients were in relapse and received therapy during the period of study, including nitrogen mustard (5 cases), x-irradiation (1 case), prednisone (2 cases), prednisone and nitrogen mustard (1 case), and x-irradiation and nitrogen mustard (1 case).

The average alpha 2 and gamma globulin levels of the sera from the patients in relapse were significantly less than the corresponding normal values. The average gamma globulin values of the sera from the patients in remission was significantly decreased below normal levels. There was no significant statistical difference between the serum protein distribution and concentration of the patients in relapse and of those in remission.

The distribution of the isoagglutinin titers in patients with malignant lymphoma is shown in Fig. 1, B. The lower titers predominated. There was no correlation between isoagglutinin titers and gamma globulin levels.

One patient with severe hypogammaglobulinemia had consistent anti-B titers of 1:2. At this time, the patient had pneumonia which persisted for 6 weeks. Following this episode, the gamma globulin rose to a normal level, although the titers did not change.

One patient was excluded from the statistical analysis. This patient (R. S.), a 60-year-old white man with lymphocytoma, exhibited a homogeneous serum component in the gamma region (Fig. 2). No titers were performed in this case.

Chronic Lymphocytic Leukemia.—The sera from 13 patients with chronic lymphocytic leukemia were studied. Four patients were in remission. Nine

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*The prednisone (Meticorten) used in this study was kindly supplied by the Schering Corporation, Bloomfield, N. J.

blue, washed in 0.5 per cent acetic acid (v/v) for 10 minutes, and fixed for 30 minutes in a solution of 0.5 per cent sodium acetate in 2 per cent acetic acid (w/v). The strips were dried in an oven at 45° C. once more, and scanned on Spinco Analytrol charts, utilizing a Spinco Model RB Analytrol to determine the percentage contribution of each protein fraction. Because of the nonlinear relationship between dye concentration and dye uptake,[30] and the nonlinear relationship between dye concentration on paper and scanner response,[31] a correction factor for the albumin had to be applied. This was determined by eluting the desired segments of the strips from the sera in 0.01 N sodium hydroxide as outlined by Crook and associates.[31] Under our conditions, this correction factor for the albumin was 1.5.

Total protein was determined by the biuret method[32] using a commercial standard protein solution* as a control.

The blood groups of the normal controls and patients were determined by using both known cells and commercial antisera.† Isoagglutinin titers were determined by the serial dilution of 0.1 ml. of serum with equal volumes of normal saline in serologic tubes. Fresh known group A and/or B cells, depending on the subject's blood type, in the amount of 0 1 ml., were added to each tube, allowed to stand for one hour at room temperature, and centrifuged at 1,500 r.p.m. for 2 minutes. The tubes were then shaken gently, and checked for macroscopic agglutination. The greatest dilution in which agglutination of the red cells occurred was taken as the end point and reported as the titer. It became apparent early in the study that many titers were undetectable at the 1:2 level of dilution. An initial tube, consisting of undiluted serum, was used thereafter, and the other dilutions carried out as described.

RESULTS

In the interpretation of the data to be presented, 3 points should be kept in mind. First, the concentration of each serum protein fraction is expressed in terms of per cent of the total protein rather than as grams per cent of plasma. Because results are expressed in this manner, any change in the concentration of one fraction will lead to complementary changes in the percentage concentration of the other fractions. Second, the electrophoretic data shown in the various tables represent uncorrected values for all constituents. This was done to avoid the inaccuracy involved in using a correction factor for abnormal globulins whose affinity for dye is unpredictable. Third, in the compilation of the isoagglutinin titers, the anti-A titers from patients and controls of groups O and B were considered as one group. Similarly, the anti-B titers from patients and controls of groups O and A were grouped together. Since there was no change in isoagglutinin titers with treatment in any of the diseases studied, and there was no difference between the titers of the patients in remission and those of the patients in relapse, the titers of all patients in each disease were considered graphically as one group.

In every disease state the observations on patients in relapse who were being treated and the observations on patients in remission who were untreated were compared with the controls and with each other. The significance of the comparisons was measured by the "t" test.[33] Significant results are specifically noted, and the direction of change is indicated.

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*Pro-Sol, obtained from Standard Scientific Corporation, Flushing, N. Y.

†Obtained from Dade Reagents, Incorporated, Miami, Fla.

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The average alpha-2 and gamma globulin levels of the sera from the patients in relapse were significantly less than the corresponding normal values. The average gamma globulin values of the sera from the patients in remission was significantly decreased below normal levels. There was no significant statistical difference between the serum protein distribution and concentration of the patients in relapse and of those in remission.

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One patient was excluded from the statistical analysis. This patient (R. S.), a 60-year-old white man with lymphocytoma, exhibited a homogeneous serum component in the gamma region (Fig. 2). No titers were performed in this case.

Chronic Lymphocytic Leukemia.—The sera from 13 patients with chronic lymphocytic leukemia were studied. Four patients were in remission. Nine patients were in relapse and received varying therapy including CB1348* (2 cases), x-ray (1 case), prednisone (2 cases), and chlorambucil and nitrogen mustard (1 case).

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*The prednisone (Meticorten) used in this study was kindly supplied by the Schering Corporation, Bloomfield, N. J.

In relapse, there were significantly increased alpha-1 globulin levels and significantly decreased gamma globulin values, while in remission there was a significant decrease in the gamma globulin fraction alone. Comparison of the sera from the patients in relapse and those in remission revealed no significant differences between the two groups.

Six patients had gamma globulin levels of less than 5 per cent. Five of these patients suffered severe respiratory infections during the course of the study. One of these patients had both pneumonia and pyelonephritis. In this latter case, the gamma globulin returned to normal levels following the subsidence of the infection

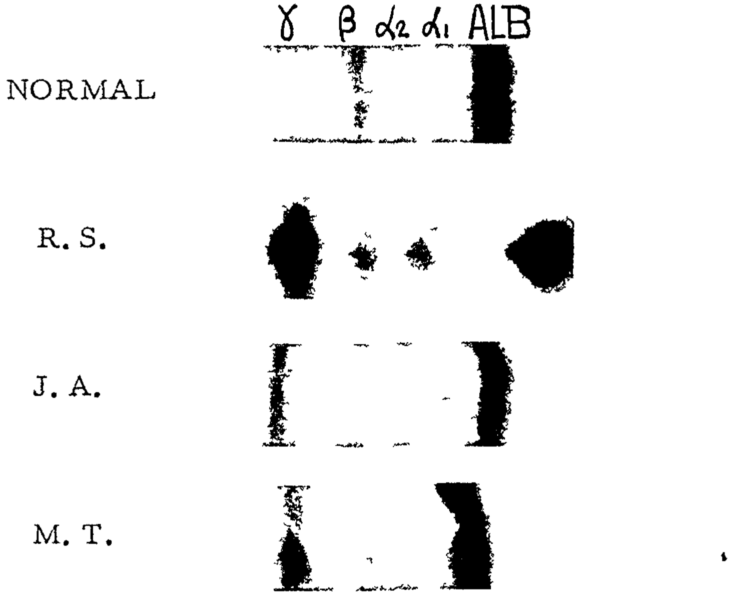

Fig. 2.—Paper electrophoretic patterns of the serum proteins of 3 patients with lymphocytoma (R. S.), chronic lymphatic leukemia (J. A.), and polycythemia vera (M. T.), respectively, showing an abnormal gamma component, which is frequently observed in multiple myeloma.

The isoagglutinin titers in patients with chronic lymphocytic leukemia are graphically shown in Fig. 1, C. The titers are considerably lower than those of the normal subjects. Although this group showed both hypogammaglobulinemia and decreased isoagglutinin titers, there was no correlation between these two factors in the individual case.

One patient was excluded from the statistical analysis. This patient (J. A.), a 60-year-old white man, had been diagnosed as chronic lymphocytic leukemia 5 years previously. He had a persistent sharp myeloma-like peak in the gamma region (Fig 2) There was no associated proteinuria Cryoglobulins were not present He had recurrent bouts of bronchitis and one episode of pneumonia Terminally, he developed oral moniliasis His anti A and anti B titers were 1 1 on several occasions Postmortem examination revealed widespread visceral involvement by leukemic infiltrates Sections were stained with phloxine methylene blue and methyl green pyronine Y (Pappenheim Saathof method) [34] No plasma cells were noted The leukemic cells showed no affinity for the pyronine

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*The chlorambucil used in this study was kindly furnished as p-di(2-chloroethyl) aminophenylbutyric acid by Burroughs Wellcome & Co., Inc., Tuckahoe, N. Y.

Chronic Myelogenous Leukemia—The sera from 13 patients with chronic myelogenous leukemia were studied Twelve patients were in relapse and received therapy during the course of study Ten patients were treated with Myleran* and two cases received prednisone

The electrophoretic and statistical data are presented in Table III In relapse, the alpha 2 and gamma globulin levels of the sera were significantly decreased, while there was a significant increase in the beta globulin fraction Repeated observation of the serum from the patient in remission revealed no significant abnormalities When the sera from the patients in relapse were compared with that of the patient in remission only one significant difference a decrease in the alpha 2 globulin fraction in the former group, was noted

The isoagglutinin titers in chronic myelogenous leukemia followed a normal distribution

Myeloid Metaplasia—Five patients with myeloid metaplasia were studied Four patients were in remission One patient received local x irradiation during the period of study

The electrophoretic and statistical data are presented in Table IV The serum of the patient in relapse was significantly decreased in its alpha 2 and beta globulin levels and significantly increased in its gamma globulin level on repeated observation The 4 patients in remission had decreased serum alpha 2 and gamma globulin values When the serum of the patient in relapse was compared with that of the patients in remission there was a significant decrease in the beta globulin fraction and a significant increase in the gamma globulin level

The isoagglutinin titers in myeloid metaplasia were low but the number of determinations was insufficient to render definite conclusions

Polycythemia Rubra Vera—The sera from 4 patients in this group were studied There was no significant difference between the control sera and patient sera The isoagglutinin titers followed the normal distribution

One patient (M T), a 52 year old Negro woman, was excluded from the statistical analysis because she had a sharp persistent myeloma like peak in the gamma region (Fig 2) Polycythemia had been diagnosed in 1954 Liver function tests were normal There was no proteinuria Cryoglobulins were not present Bone marrow aspirations on 3 recent occasions revealed hypocellular marrow with a shift to the left There was no increase in plasma cells The anti A and anti B titers were 1 32 and 1 16, respectively, on several occasions

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*Busulfan (1 4 dimethanesulfonyloxybutane)

TABLE III. SERUM PROTEIN DISTRIBUTION AND CONCENTRATION IN PATIENTS WITH CHRONIC MYELOGENOUS LEUKEMIA

	VALUES	NUMBER OF PATIENTS	NUMBER OF OBSERVATIONS	ALBUMIN (%)	ALPHA-1 GLOBULIN (%)	ALPHA-2 GLOBULIN (%)	BETA GLOBULIN (%)	GAMMA GLOBULIN (%)	TOTAL PROTEIN (GM. %)
Group 1 patients in relapse	Mean	12	36	47.2	5.8	11.7	16.7	18.7	6.2
	Standard deviation			10.8	1.9	2.2	4.2	2.0	0.8
Group 2 patients in remission	Mean	1	3	43.0	5.3	13.4	17.1	20.5	5.74
	Standard deviation			7.7	0.6	0.8	2.9	5.9	1.45
Patients in relapse compared with controls	Significance (p)			0.9	1.0	0.02 ↓*	0.001↑	0.05↓	0.40
Patients in remission compared with controls	Significance (p)			0.4	0.1	0.5	0.2	0.9	0.4
Patients in relapse compared with patients in remission	Significance (p)			0.4	0.2	0.005↓	0.9	0.5	0.5

*See footnote to Table II

TABLE IV SERUM PROTEIN DISTRIBUTION AND CONCENTRATION IN PATIENTS WITH MYELOID METAPLASIA

	VALUES	NUMBER OF PATIENTS	NUMBER OF OBSERVATIONS	ALBUMIN (%)	ALPHA 1 GLOBULIN (%)	ALPHA 2 GLOBULIN (%)	BETA GLOBULIN (%)	GAMMA GLOBULIN (%)	TOTAL PROTEIN (GM %)
Group 1 (patients in relapse)	Mean	1	4	48.3	5.4	7.8	11.6	24.3	6.3
	Standard deviation			6.29	1.8	2.1	0.8	1.6	0
Group 2 (patients in remission)	Mean	4	10	51.9	6.6	8.2	15.5	15.5	6.4
	Standard deviation			9.9	1.9	2.8	4.0	4.2	0.7
Patients in relapse compared with controls	Significance (p)			0.7	0.6	0.001↓*	0.001↓	0.001↑	0.3
Patients in remission compared with controls	Significance (p)			0.1	0.3	0.001↓	0.6	0.003↓	0.9
Patients in relapse compared with patients in remission	Significance (p)			0.4	0.3	0.8	0.003↓	0.001↑	0

*See footnote to Table II

TABLE V. SERUM PROTEIN DISTRIBUTION AND CONCENTRATION IN PATIENTS WITH ACUTE LEUKEMIA

	VALUES	NUMBER OF PATIENTS	NUMBER OF OBSERVATIONS	ALBUMIN (%)	ALPHA-1 GLOBULIN (%)	ALPHA-2 GLOBULIN (%)	BETA GLOBULIN (%)	GAMMA GLOBULIN (%)	TOTAL PROTEIN (GM. %)
Group 1 (patients in relapse)	Mean	4	7	35.1	7.5	14.6	13.1	29.3	5.8
	Standard deviation			10.2	1.0	3.3	2.1	8.6	0.7
Group 2 (patients in remission)	Mean	6	17	52.8	5.5	10.7	14.4	16.9	6.8
	Standard deviation			9.5	1.8	3.3	3.9	5.2	1.0
Patients in relapse compared with controls	Significance (p)			0.02 ↓*	0.005↑	0.3	0.08	0.02 ↑	0.01
Patients in remission compared with controls	Significance (p)			0.02 ↑	0.3	0.01↓	0.5	0.03 ↓	0.4
Patients in relapse compared with patients in remission	Significance (p)			0.001↓	0.001↑	0.02↓	0.3	0.005↑	0.06

*See footnote to Table II.

Acute Leukemia.—The sera from 10 patients with acute leukemia were studied, including acute lymphocytic leukemia (6 cases), acute monocytic leukemia (3 cases), and histiocytic leukemia (1 case). Four patients were treated with prednisone, and two patients received Meticorten and 6-mercaptopurine.

The electrophoretic and statistical data are presented in Table V. In relapse, there were significantly decreased serum albumin and total protein levels and significantly increased alpha 1 and gamma globulin values. In remission, there were significantly elevated serum albumin values and significantly decreased values of alpha 2 and gamma globulin. When the sera from the patients in relapse were compared with that of the patients in remission, the albumin and alpha 2 globulin fractions were significantly lower and the alpha 1 and gamma globulin fractions were significantly higher in the former group.

There was no difference between the isoagglutinin titers of the 10 patients with acute leukemia and those of the control group. Moreover, there was no correlation between gamma globulin levels and isoagglutinin titers in this group.

Multiple Myeloma.—Twelve patients with multiple myeloma were studied, including one case of plasma cell leukemia. Eight patients received therapy including urethane (6 cases), x-ray (1 case), and prednisone (1 case). Ten patients had homogeneous serum constituents with the following mobilities: 4 cases, fast gamma, 3 cases, intermediate gamma, 2 cases, slow gamma, and 1 case, slow and fast gamma.

The electrophoretic and statistical data for this group are presented in Table VI. The 4 patients in relapse had significantly decreased serum levels of albumin and significantly elevated values for the alpha 1 and gamma globulin fractions. The 8 patients in remission had significantly low serum albumin and beta globulin values and significantly elevated values for the gamma globulin and total protein concentration. When the sera from the patients in relapse were compared with that of the patients in remission, the values for the albumin fraction and total protein concentration were significantly lower, and the levels of the alpha 1 and gamma globulin fractions were significantly higher in the former group.

In 3 cases, treatment was of such short duration that the effects could not be evaluated. X-irradiation to localized lesions and Meticorten produced no effect on the protein distribution. There was a decrease in the height of the peak in 4 cases treated with urethane. One patient had a decreased peak initially under therapy which subsequently increased. In another (M. C.), the band disappeared completely from the tracings of a sample taken 25 days after urethane therapy. Technical and clerical errors appeared to be satisfactorily ruled out as an explanation for this striking change. Electrophoresis two months later revealed reappearance of the band (Fig. 3, C). Unfortunately, intermediate sera were not available. Urethane therapy produced a considerable decrease in the alpha 1, beta, and gamma globulins, and

Table VI. Serum Protein Distribution and Concentration in Patients With Multiple Myeloma

	VALUES	NUMBER OF PATIENTS	NUMBER OF OBSERVATIONS	ALBUMIN (%)	ALPHA-1 GLOBULIN (%)	ALPHA-2 GLOBULIN (%)	BETA GLOBULIN (%)	GAMMA GLOBULIN (%)	TOTAL PROTEIN (GM. %)
Group 1 (patients in relapse)	Mean	4	12	30.6	9.3	12.1	12.7	35.7	6.2
	Standard deviation			7.3	2.7	3.1	3.4	6.1	0.4
Group 2 (patients in remission)	Mean	8	19	42.6	5.8	12.0	12.3	25.8	7.3
	Standard deviation			7.2	2.1	5.3	6.5	8.4	1.7
Patients in relapse compared with controls	Significance (p)			0.001↓*	0.005↑	0.5	0.1	0.001↑	0.5
Patients in remission compared with controls	Significance (p)			0.01 ↓	0.7	0.4	0.04↓	0.003↑	0.03↑
Patients in relapse compared with patients in remission	Significance (p)			0.001↓	0.005↑	1.0	0.8	0.005↑	0.03↓

*See footnote to Table II

a significant elevation of the albumin fraction in 3 of 6 cases. There was a concurrent decrease in the total protein concentrations. With continued urethane therapy in 2 of these 3 cases, the albumin once more decreased and was accompanied by a rise in the alpha 1, beta, and gamma globulins, and total protein concentration.

The distribution of the isoagglutinin titers is shown in Fig. 1, D. There was a striking decrease in the titers in multiple myeloma. Correlation between the presence or absence of abnormal protein components with isoagglutinin titers could not be determined because of the limited number of cases without these proteins.

Four patients had pneumonia during the period of study, while one patient succumbed to a staphylococcal septicemia which arose in a buttock abscess.

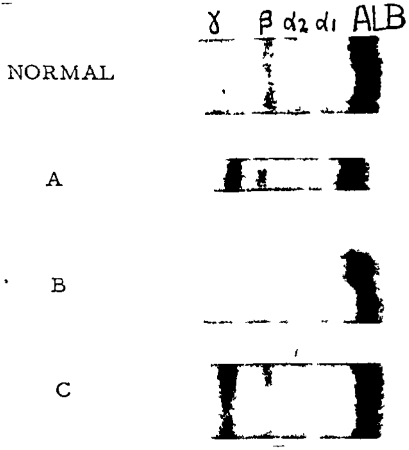

Fig. 3.—Paper electrophoretic patterns of the serum proteins of a patient (M. C.) with multiple myeloma exhibiting (A) an intermediate gamma band prior to urethane therapy, (B) disappearance of the myeloma protein with urethane therapy, and (C) the reappearance of the abnormal component on continued therapy.

DISCUSSION

Our results are in general agreement with those of other investigators. Abnormalities are frequently present in the serum of patients with diseases of the reticuloendothelial system, the gamma globulins being most frequently altered.

It is interesting to note that the findings in Hodgkin's disease differ in several respects from other types of malignant lymphoma and chronic lymphatic leukemia. First, hypoalbuminemia is a significant finding in the patients with Hodgkin's disease in relapse, while this is not so in the other groups studied. Although this may simply be a nonspecific response to the severity of the illness, the case material does not give support to this theory. Second, there was an increase in the alpha and beta globulins with a normal gamma value in this same group of patients, while those with lymphoma and lymphatic leukemia had variable changes in alpha globulins associated with a significant decrease in gamma globulins. Third, the isoagglutinins were present in normal titer, while those in the lymphoma-lymphatic leukemia group were low.

These findings differ from some of the reported studies of electrophoresis in Hodgkin's disease, which have frequently revealed an increase in gamma globulin.[1-3, 7, 35] Also, in contrast to other reports of serum electrophoresis in cases of lymphoma which have described an increase in alpha, beta, and gamma globulins, a significant decrease in gamma globulin was demonstrated in the present series. Our observations of hypogammaglobulinemia in lymphatic leukemia, however, do confirm observations of others.[2, 4, 6, 9] The discrepancy between our results and those of other investigators may be partially explained by the difference in separation of protein fractions in the Tiselius apparatus and by the clinical status of the patients themselves.

Studies of isoagglutinins in the lymphoma-lymphatic leukemia group revealed a definite decrease in titer, and although there was no direct correlation with total gamma globulin, this finding was coincident with the tendency toward hypogammaglobulinemia.

In chronic myelogenous leukemia, serum protein changes were also found. Both alpha-2 and gamma globulins were significantly decreased, and beta globulin increased, in agreement with other reports.[1, 2, 4, 6, 9]

The other myeloproliferative diseases studied varied somewhat from the leukemic group. The alpha-2 and beta globulins were significantly decreased, and gamma globulins were normal or low in myeloid metaplasia, while no abnormalities were found in polycythemia vera. This may merely reflect the extensiveness of the extramedullary involvement in these syndromes. Although gamma globulins were abnormal in patients of the myeloproliferative category, isoagglutinin titers did not reflect these abnormalities, but showed a normal distribution.

Acute leukemia is also associated with significant serum protein alterations. The presence of hypoalbuminemia, hypoproteinemia, and increased gamma globulin in the present series confirms the results of other investigators.[5, 6, 10, 11, 35] These changes revert toward normal during remission.

Our results in multiple myeloma are also similar to those of many authors.[14-26] Hypoalbuminemia is associated with an elevation of alpha-1 and gamma globulins and an increase in total protein concentration. All the myeloma proteins found had mobilities in the gamma area. It should be noted, however, that myeloma proteins may occur in the range from alpha-2 through slow gamma.

Therapy led to a partial reversion of the protein pattern to normal with improvement of the clinical status. Such a change has also been reported by

Rundles and associates[36, 37] The complete, although transitory, disappearance of the myeloma protein band noted in one of our patients is, however, rare

Although gamma globulins were increased in myeloma, a decrease in isoagglutinin titers suggested that there was an actual decrease in the proportion of normal gamma globulin components

It is noteworthy that homogeneous serum components may also be found in diseases other than myeloma[8, 14, 38, 44] In the present series, 3 cases (1 with lymphocytic lymphoma, 1 with chronic lymphocytic leukemia, and 1 with polycythemia vera) had peaks in the gamma range resembling myeloma protein One might postulate that the malignant lymphocyte is the source of this protein in the first 2 cases, since the close relationship between plasma cell lymphocytes, and gamma globulins has been emphasized by many[38, 48, 56] The source of the abnormal protein in the third case remains unexplained

Significantly decreased isoagglutinin titers occurred in our patients with malignant lymphoma, chronic lymphocytic leukemia, and multiple myeloma Schiff and Mendlowicz[57] were the first investigators to record the frequency of very low isoagglutinin titers in leukemia Zuendel[58] corroborated these observations in another series of leukemic patients He also noted that titers increased with remission of the disease Davidsohn studied 38 patients with acute and chronic leukemia and found that the titers in the chronic cases were less than those of a control group He noted no difference between myelocytic and lymphocytic leukemia, but he found both to be more significantly decreased than the acute cases It is possible that the tendency toward low values could be partially explained in our series by the older age incidence in the lymphocytic leukemia and myeloma group, since the titers are said to decline with age[59] However, the trend was not confined to older patients

Several patients with hypogammaglobulinemia suffered bacterial infections during the course of the study The association of the lack of immune response with recurrent infections in patients with malignant disorders of the hematopoietic and reticuloendothelial system has been emphasized,[60-66] and the low titers of isoagglutinins in some of these diseases may be considered as another manifestation of the inadequate immune mechanisms often present in these disorders Recognition of this defect could certainly be of some clinical significance in the management of these cases

SUMMARY

The serum proteins in 99 patients with Hodgkin's disease, other malignant lymphomas, acute leukemia, chronic lymphocytic leukemia, the myeloproliferative disorders, and multiple myeloma have been studied by electrophoretic and serologic techniques The results of this analysis have been described, and the significant alterations commented upon together with observations on the effects of therapy In addition, the occurrence of myeloma like proteins in the sera of 3 patients with lymphoblastoma, chronic lymphocytic leukemia, and polycythemia rubra vera is reported

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THE ACID BASE BALANCE AND SODIUM DISTRIBUTION OF THE BLOOD IN ESSENTIAL HYPERTENSION

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ACUTE alterations of the partial pressure of carbon dioxide within the pulmonary alveoli affect the vascular system Respiratory acidosis produced by the inhalation of carbon dioxide causes an elevation of both systolic and diastolic blood pressures of normal man[1] The normal dog and the renal hypertensive dog have a similar pressor response, although the neurogenic hypertensive dog and the curarized dog show a depressor effect[2]

The converse of acute hypercapnea causing elevation of the blood pressure has also been reported, that is, it has been found that in essential hypertension there is a primary respiratory acidosis[3] It is possible that this may be related to the alteration in the serum sodium concentration which has been reported in this disease[4, 5] These considerations prompted further study of the acid base balance and the plasma and whole blood sodium and water contents of patients having essential hypertension Preliminary studies tended to confirm the reported association of respiratory acidosis with essential hypertension* Further evaluation has failed to substantiate this correlation

MATERIALS AND METHODS

Eleven normotensive subjects, 8 laboratory workers and 3 patients (9 men and 2 women), with resting blood pressures below 145/90 mm Hg and 26 patients (16 men and 10 women) with essential hypertension having blood pressures above these levels were studied Five of the hypertensive subjects were receiving antihypertensive drugs (3 reserpine and 2 mecamylamine) None had clinical or laboratory evidences of pulmonary disease, cardiac failure or renal insufficiency Arterialized, heparinized venous blood was withdrawn from the heated arm after 30 minutes of recumbency and the plasma separated anaerobically Blood pressure readings were taken before and after venesection The mean blood pressure was calculated as the diastolic pressure plus one third of the pulse pressure Plasma pH,[6] CO content,[7] and the per cent of packed red cells were determined, and the partial pressure of carbon dioxide (pCO_2), the plasma bicarbonate concentration, and the whole blood buffer base concentration (B_B^+) were calculated[8] The water content of both plasma and whole blood was determined by drying to constant weight and the sodium concentration by flame photometry

RESULTS

The mean blood pressure of the normal group was 90 mm Hg and that of the hypertensive patients was 146 mm Hg (Table I) In the hypertensive

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*Abstracted in the Proceedings of the Central Society for Clinical Research J Lab & Clin Med 50 964 1957 group the male patients had a mean pressure of 148 mm. Hg and the female subjects had a mean of 142 mm. Hg. The hypertensive patients had a lower mean hematocrit value than did the normotensive subjects. This was not due to the proportionally greater number of female patients in this group because the mean hematocrit values for each sex were nearly the same. The plasma pH was similar in both groups, being 0.02 pH unit lower in the hypertensive patients. There were virtually no differences in the mean plasma CO_2 contents or calculated bicarbonate concentrations. The whole blood buffer base concentration (B_B+) was slightly lower in the hypertensive group because of the lower per cent of red cells present. The partial pressure of carbon dioxide in the arterialized blood (pCO_2) was slightly higher in the hypertensive group. In the normotensive group the male subjects had a mean pCO_2 of 44.9 mm. Hg and the females 43.9 mm. Hg. In the hypertensive group the male patients had a mean pCO_2 of 44.9 mm. Hg and the females 46.4 mm. Hg.

TABLE I. MEAN DETERMINED AND DERIVED VALUES DESCRIBING THE ACID-BASE BALANCE

GROUP	MEAN BLOOD PRESSURE (mm. Hg)	HEMATOCRIT (PER CENT)	PLASMA pH (UNITS)	PLASMA CO_2 CONTENT (mM./L.)	WHOLE BLOOD (B_B+) (mEq /L.)	pCO_2 (mm. Hg)
Normal						
Mean	90	47.8	7.38	26.84	48.4	44.7
S.E.		1.08	0.009	0.432	0.379	1.13
Hypertensive						
Mean	146	44.2	7.36	26.89	47.6	45.5
S.E.		0.96	0.009	0.511	0.384	1.31

S.E. = Standard error of the mean.

TABLE II. MEAN DETERMINED AND DERIVED VALUES DESCRIBING SODIUM CONTENT AND DISTRIBUTION

GROUP	PLASMA SODIUM (mEq./L.)	PLASMA WATER (GM./100 GM.)	WHOLE BLOOD SODIUM (mEq./L.)	WHOLE BLOOD WATER (GM./100 GM.)	RED CELL SODIUM (mEq./L. R.B.C.)
Normal					
Mean	143	91.8	92	79.1	35
S.E.	1.22	0.75	2.68	0.75	6.24
Hypertensive					
Mean	142	91.2	93	80.3	34
S.E.	1.30	0.15	2.19	0.49	6.08

The mean plasma sodium concentration of the hypertensive group was 1 mEq. lower than that of the normotensive subjects whereas the mean whole blood sodium concentration was 1 mEq. higher (Table II). The calculated sodium content per liter of red cells was slightly lower in the hypertensive patients. The plasma water content was nearly the same in both groups. The whole blood water content was slightly higher in the hypertensive group leading to a greater calculated cell water content per liter of red cells. When the sodium concentration of the plasma and red cells is calculated per liter of water in each phase, the hypertensive group had a lower intracellular sodium concentration.

None of the differences between group means in the parameters of the acid-base balance or in sodium distribution was statistically significant. Graphic presentation of these determinants disclosed no correlations with the blood pressure.

DISCUSSION

Waldron and Goldstein reported that the mean pH of the arterial blood of 19 hypertensive patients without renal functional impairment was 0.05 unit lower than that of 14 normotensive subjects and their mean partial pressure of carbon dioxide (pCO_2) was nearly 8 mm. Hg higher.[3] Although these differences in pH and pCO_2 between groups were statistically significant, all of these values fall within the normal range as defined by Singer and Hastings.[8] From the data of Waldron and Goldstein, assuming the pH's to be corrected to body temperature and the normal arterial oxygen capacity to be 19.0 vol. per cent with an hematocrit of 45 per cent, the mean whole blood buffer base is calculated to be 48.6 mEq./L. in the normotensive subjects and 47.5 mEq./L. in the hypertensive patients, both being normal values.

These findings in the hypertensive group of a lower pH, a higher pCO and an unaltered buffer base are in the direction of those present in a primary respiratory acidosis but without any associated metabolic deviation in the acid-base balance. The data reported in this study are in agreement with the findings of Waldron and Goldstein as to direction of change but the differences between groups are of lesser magnitude and lack statistical significance.

Many factors influence the carbon dioxide tension and pH of the arterial blood. Variations due to diet, activity, and age were eliminated in the study of Waldron and Goldstein. Sex distribution was not specified. It has been found that the CO_2 tension of the blood is 4 mm. Hg less in women than in men.[9] In the series presented here this relationship was not found in the hypertensive patients. The pCO_2 of the female patients was slightly higher than that of the male subjects. This suggests that the female hypertensive patients do have a tendency toward a primary respiratory acidosis while the male subjects with hypertension do not. From this evidence it would appear that there is no direct relationship between the primary respiratory acidosis and the presence of essential hypertension per se.

The acid-base balance of the blood is very sensitive to changes in respiratory activity. During sleep the carbon dioxide tension of the blood rises about 7 mm. Hg.[10] Similarly a primary respiratory acidosis ensues quite promptly following initiation of breathing into a closed system.[11] In such acute situations there is no associated metabolic alteration in the blood buffer base. If a primary change in the acid-base balance persists long enough a secondary change in the other variable does occur. Such is the case in pulmonary emphysema where the primary respiratory acidosis is accompanied by a secondary metabolic alkalosis.[8] The absence of a secondary alteration in the blood buffer base in the hypertensive patients studied by Waldron and Goldstein suggests that the tendency toward a primary respiratory acidosis was of relatively short duration and therefore likely due to a transient physiologic disturbance rather than to pulmonary disease. Indeed these authors state that their patients were free of any pathologic condition which might interfere with normal pulmonary ventilation.[3] In the hypertensive subjects reported in the present study there was likewise no evidence of pulmonary emphysema or any other pulmonary disease.

The hypernatremia reported to be present in hypertensive patients as a group was not demonstrated in this study. Holley and Holland found the mean plasma sodium concentration of 75 hypertensive patients to be 2.5 mEq./L. higher than that of 400 normotensive subjects.[4] Albert and coworkers reported that the plasma sodium concentration was nearly 6 mEq./L. higher in hypertensive subjects.[5] A previous study from this laboratory suggested that the milder cases of essential hypertension had slightly elevated plasma sodium concentrations.[12] This was not evident in the survey presented here.

SUMMARY

A study of 26 patients having essential hypertension and 11 normotensive subjects failed to demonstrate a direct relationship between elevation of the blood pressure and the presence of a primary respiratory acidosis or hypernatremia. The tendency of hypertensive patients as a group to have a respiratory acidosis, as evidenced by the slight elevation of the partial pressure of carbon dioxide and lowering of the pH of their blood, but without any change in buffer base, is most likely the result of transient physiologic hypoventilation.

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ESTIMATION OF BILIRUBIN MONO AND DIGLUCURONIDE IN THE PLASMA AND URINE OF PATIENTS WITH NONHEMOLYTIC JAUNDICE

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EVIDENCE from several laboratories indicates that the direct reacting bilirubin in plasma is a mixture of two principal compounds, bilirubin mono and diglucuronide[1-4] The sole method available for estimating the bilirubin conjugates is the column chromatographic assay described by Billing[1] With this technique Bollman[3] has observed that the estimation of bilirubin mono and diglucuronide in plasma can be of value in the differential diagnosis of jaundice Therefore, it seemed worth while to develop a more simple, rapid, and sensitive procedure for estimating the mono and diglucuronide in direct reacting bilirubin

In the method described below the relatively labile bilirubin glucuronides are initially coupled with diazotized sulfanilic acid (the direct van den Bergh reaction) to yield the stable azopigment derivatives In the reaction 1 mole of diglucuronide yields 2 moles of azopigment B, and 1 mole of monoglucuronide yields 1 mole of azopigment A plus 1 mole of azopigment B[2] Subsequently, the azopigments are extracted, separated by a simple partition between butanol chloroform and acetate buffer, and estimated colorimetrically The proportions of mono and diglucuronide in the direct reacting bilirubin are calculated from the relative quantities of azopigments A and B found

The method has been applied to estimations of the mono and diglucuronide in the direct reacting bilirubin in human T tube bile, and in the plasma and urine of patients with nonhemolytic jaundice In addition, the renal clearances of bilirubin mono and diglucuronide have been determined simultaneously in each of 4 patients

MATERIALS AND METHODS

Preparation of Azopigments—Solutions of purified azopigments A and B were prepared as reference standards for the procedure Azopigment A was prepared by coupling a chloroform solution of pure bilirubin (Mann Research Laboratories, m p 200° C, λ max 450 mμ in chloroform, $E = 53 6 \times 10^3$) with diazotized sulfanilic acid in 50 per cent methanol[5] The resulting solution of azopigment A was evaporated at 40° to 50° C almost to dryness and diluted with water to a convenient volume Azopigment B was prepared by treating fresh human T tube bile with diazotized sulfanilic acid Subsequently, the azopigment B was extracted with *n* butanol and purified by ascending paper chromatography[6] Alternatively, an equal volume of 2 0 M acetate of pH 3 0 was added

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physiologic disturbance rather than to pulmonary disease. Indeed these authors state that their patients were free of any pathologic condition which might interfere with normal pulmonary ventilation.[3] In the hypertensive subjects reported in the present study there was likewise no evidence of pulmonary emphysema or any other pulmonary disease.

The hypernatremia reported to be present in hypertensive patients as a group was not demonstrated in this study. Holley and Holland found the mean plasma sodium concentration of 75 hypertensive patients to be 2.5 mEq./L. higher than that of 400 normotensive subjects.[4] Albert and coworkers reported that the plasma sodium concentration was nearly 6 mEq./L. higher in hypertensive subjects.[5] A previous study from this laboratory suggested that the milder cases of essential hypertension had slightly elevated plasma sodium concentrations.[12] This was not evident in the survey presented here.

SUMMARY

A study of 26 patients having essential hypertension and 11 normotensive subjects failed to demonstrate a direct relationship between elevation of the blood pressure and the presence of a primary respiratory acidosis or hypernatremia. The tendency of hypertensive patients as a group to have a respiratory acidosis, as evidenced by the slight elevation of the partial pressure of carbon dioxide and lowering of the pH of their blood, but without any change in buffer base, is most likely the result of transient physiologic hypoventilation.

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petroleum ether for 2 minutes. The azopigment A was re-extracted into the 4.0 ml. of the aqueous (lower) layer, resulting in a fourfold increase in concentration. The optical density at 540 mμ of the azopigment A-containing phase was estimated after centrifugation.

Calculations.—The fraction (M) of direct-reacting bilirubin which is monoglucuronide, or biglucuronide (1-M), can be calculated from the concentrations of azopigment in the acetate buffer layer (C_A, expressed in optical density units per milliliter) and in the butanol-chloroform layer (C_O) estimated in Step 3, by the following equation:*

$$M = 0.27/(C_A/C_O + 2.00) \qquad (1)$$

When Equation 1 is rearranged to $C_A/C_O = 0.27(1/M) - 2.00$, the relationship between C_A/C_O and 1/M can be plotted linearly as shown in Fig. 1.

Comments.—The entire procedure was routinely completed within 30 minutes to minimize possible hydrolysis of azopigment B at pH 2 to 3. The values obtained were reproducible: in 10 determinations of a sample of plasma the per cent monoglucuronide in the direct-reacting bilirubin was 66 ± 2 (mean ± standard deviation).

The direct van den Bergh reaction as performed above appears to be specific for conjugated bilirubin. This was demonstrated by dissolving pure bilirubin in a small volume of 0.2 N NaOH and adding it to normal plasma. With concentrations of indirect-reacting bilirubin as high as 25 mg. per cent, the quantities of azopigment A formed in the direct reaction were insignificant.

RESULTS

Comparison With the Chromatographic Method.—The results of estimations of the per cent of mono- and diglucuronide in the direct-reacting bilirubin of bile, urine, and plasma are shown in Fig. 1, and agree with the values obtained by others with the column chromatographic method.[1,3,4] Bilirubin diglucuronide accounted for 84 and 81 per cent of the direct-reacting bilirubin in the bile and urine specimens, respectively. In contrast, bilirubin monoglucuronide was frequently the major fraction in the plasmas of the 23 patients with nonhemolytic jaundice. The mean value in plasma was 61 per cent monoglucuronide, comparable to 65 per cent monoglucuronide (19 observations, 17 patients) and 52 per cent (59 observations, 22 patients) reported in two studies with the chromatographic method.[1,4]

A sample of plasma from each of 10 patients was tested by both the chromatographic and the present techniques to compare the methods directly. The values obtained paralleled each other with a correlation coefficient of 0.84 ($p < 0.01$). The proportion of monoglucuronide was generally 10 to 15 per cent lower with the chromatographic method.†

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*$M = A/(A + B)$ where A = total azopigment A in the acetate/butanol-chloroform tube = $(C_O)(6.0)(1/0.89)$ and B = total azopigment B in the tube = $\frac{(C_A)(1.5) - (A)(0.11)}{(0.11)}(1/0.7)$. Substituting for A and B and simplifying results in Equation 1. The factor 1/0.7 corrects for the less complete extraction of azopigment B relative to azopigment A in the initial butanol extraction. The values for the constants in Equation 1 should be checked with standard azopigments A and B and may vary slightly.

† Billing, Cole and Lathe[4] have noted a requirement for ethanol for complete coupling of pigment I (obtained from columns) with the diazo reagent. If this were also true in plasma it would result in low values for the monoglucuronide as estimated above. However the monoglucuronide values are somewhat higher than those observed with the column method, suggesting that in plasma the bilirubin monoglucuronide may be coupled completely with diazotized sulfanilic acid in the absence of ethanol.

Plasma Bilirubin Glucuronides.—Table I summarizes the results of estimations of the per cent monoglucuronide in the plasmas of 23 jaundiced patients with various diagnoses. In each instance the diagnosis was established by history and physical examination, liver function tests, typical clinical course, and, except in the cases of probable viral hepatitis, by liver biopsy or surgical exploration. The per cent monoglucuronide ranged from 55 to 65 in 13 of the

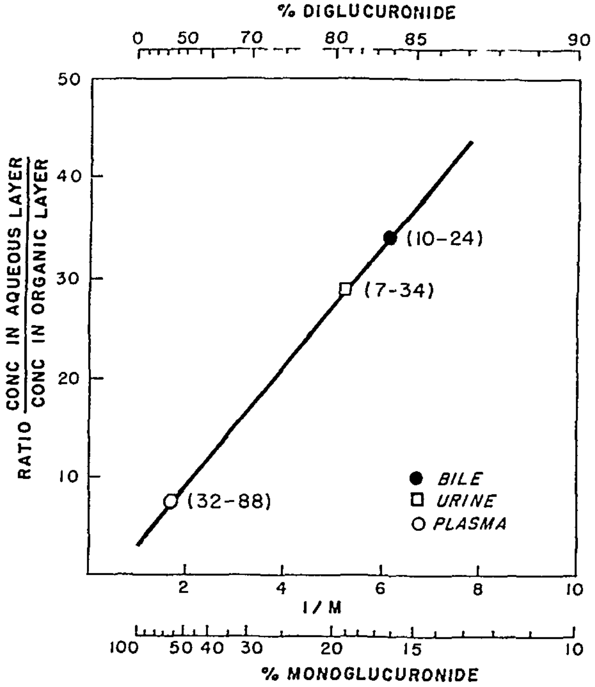

Fig. 1.—Relationship between the ratio of the concentrations of azopigments in the aqueous and organic layers and the proportions of mono- and diglucuronide in the direct-reacting bilirubin. A plot of C_A/C_O versus 1/M (see text) results in the straight line shown. The mean values (indicated on the line) and the ranges of values for the per cent monoglucuronide (figures in parentheses) in bile, urine, and plasma are shown.

TABLE I. THE PER CENT OF MONOGLUCURONIDE IN THE PLASMA DIRECT REACTING BILIRUBIN OF PATIENTS WITH NONHEMOLYTIC JAUNDICE

NUMBER OF PATIENTS	PER CENT OF TOTAL PATIENTS	PER CENT MONO GLUCURONIDE	DIAGNOSES*
4	17	32-48	Acute choledocholithiasis (1); early viral hepatitis (1); cholangiolitic hepatitis (1); hemochromatosis (1)
13	57	55-65	Viral hepatitis (9); chronic biliary obstruction [congenital atresia of bile ducts (1), carcinoma of common bile duct (1); carcinoma of head of pancreas (1)], portal cirrhosis (1)
6	26	67-88	Portal cirrhosis (2); diffuse hepatic carcinoma (2); secondary biliary cirrhosis, 8 years (1); Dubin-Johnson syndrome (1)

*The number of cases with each diagnosis is indicated by parentheses.

23 patients. This group included most of the cases of viral hepatitis and 3 patients with extrahepatic biliary obstruction for over 2 months. Relatively low monoglucuronide values (i.e., high diglucuronide levels) were observed in individual patients with acute choledocholithiasis (32 per cent monoglucuronide), viral hepatitis at the onset of jaundice (43 per cent), cholangiolitic hepatitis (48 per cent), and hemochromatosis (45 per cent). Relatively high monoglucuronide levels, ranging from 67 to 88 per cent, were observed in 6 patients, including 2 cases of portal cirrhosis (67 and 69 per cent), 2 cases of diffuse intrahepatic carcinoma (78 and 88 per cent), and 1 case of secondary biliary cirrhosis for over 8 years (74 per cent).

Renal Clearance of Bilirubin Glucuronides.—The renal clearances of bilirubin mono- and diglucuronide have been estimated simultaneously in each of 4 jaundiced patients. Timed, voided urine samples were collected for 2 to 3 hours, and venous blood samples were drawn at the mid-points of the collection periods. The value for the total direct-reacting bilirubin in each sample[2] was multiplied by the fraction of mono- and diglucuronide to yield the concentration of each conjugate in plasma and urine, and the renal clearances were calculated by conventional methods. As shown in Table II the clearances of bilirubin diglucuronide varied from 0.41 to 0.96 ml. per minute, and considerably exceeded the clearances of the monoglucuronide, 0.05 to 0.16 ml. per minute.

TABLE II. THE RENAL CLEARANCES OF BILIRUBIN MONO- AND DIGLUCURONIDE

PATIENT	DIAGNOSIS	RENAL CLEARANCE (ML. PER MINUTE)	
		MONOGLUCURONIDE	DIGLUCURONIDE
1	Viral hepatitis	0.11	0.47
2	Biliary cirrhosis	0.16	0.88
3	Viral hepatitis	0.09	0.41
4	Carcinoma of head of pancreas	0.05	0.96

DISCUSSION

Experimental and clinical studies described by Bollman indicate that bilirubin is conjugated to the diglucuronide only in the liver. The latter compound is the major bilirubin conjugate secreted into the bile and may accumulate in the blood following acute biliary obstruction. Bilirubin monoglucuronide can be synthesized in extrahepatic tissues and may accumulate in the blood in hepatectomized animals and in patients with hepatic parenchymal disease.[3] Thus, it would be anticipated that estimation of the bilirubin glucuronides in plasma could be of value in the diagnosis of jaundice. In confirmation of Bollman's observations, high plasma diglucuronide levels were observed in a patient with acute common duct obstruction and in another with viral hepatitis during the onset of jaundice. The proportion of monoglucuronide was considerably higher in patients with chronic biliary obstruction (2 months or longer), and in these patients the proportion did not differ significantly from that observed in primary hepatic parenchymal disease. The highest monoglucuronide levels were noted in patients with advanced portal cirrhosis, diffuse hepatic neoplasm, and in one case of secondary biliary cirrhosis of 8 years' duration.

It seems reasonable to conclude that a marked predominance of bilirubin diglucuronide in the plasma suggests the diagnosis of acute biliary obstruction, whereas a very high proportion of the monoglucuronide suggests hepatic parenchymal disease. Inasmuch as almost 60 per cent of the patients studied comprised an intermediate group, with monoglucuronide levels varying from 55 to 65 per cent of the direct-reacting bilirubin, estimation of the glucuronides in plasma appears to be of limited diagnostic value.

The method of estimation described can be useful in studies of bile pigment metabolism. The procedure is simple, rapid, and applicable to samples containing as little as 4 mg. per cent direct-reacting bilirubin. The values obtained are similar to those observed with the more complicated chromatographic technique. In the present studies the method has been applied to the simultaneous estimation of the renal clearances of the bilirubin glucuronides. The predominance of the diglucuronide in the urine of patients with nonhemolytic jaundice[2, 8] is associated with a considerably higher renal clearance of this conjugate as compared to the monoglucuronide.

SUMMARY

1. A simple method is described for the estimation of the mono- and diglucuronide in the direct-reacting bilirubin of plasma, urine, and bile.

2. Bilirubin diglucuronide is the major bilirubin compound in human T-tube bile. In patients with nonhemolytic jaundice the diglucuronide predominates in the urine, whereas the monoglucuronide is frequently the major conjugate in the plasma.

3. Estimation of the bilirubin conjugates in plasma is of limited diagnostic value, although high diglucuronide levels suggest acute biliary obstruction and high monoglucuronide values suggest hepatic parenchymal disease.

4. The renal clearance of bilirubin diglucuronide is considerably greater than that of the monoglucuronide.

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and 2) belonged to irreversibly anuric subjects, maintained by means of repeated treatments with the artificial kidney* Two other patients (Cases 3 and 4) were severely ill, and died soon after admission On the other hand, the lower RNase activities belonged to patients with either acute renal disease with clinical recovery (Cases 16 and 18), or to stationary chronic renal disease with relatively low concentrations of urea in the blood (Cases 15 and 17) Cases

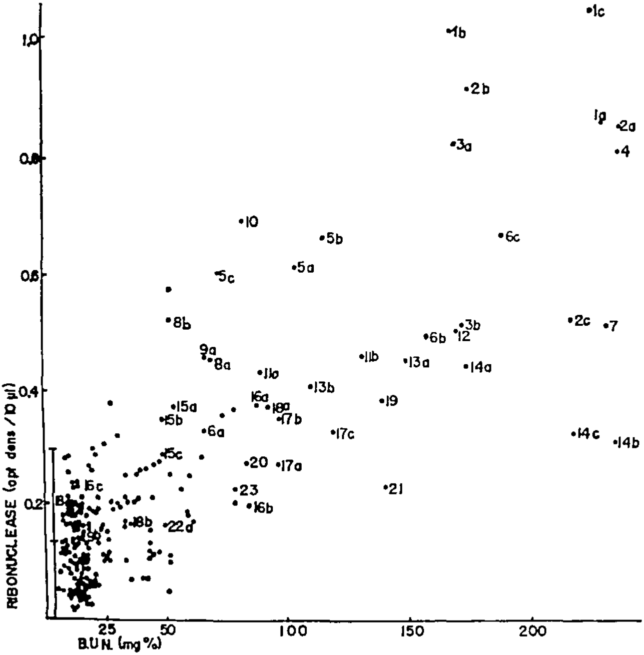

Fig 1—Plot of plasma RNase activity (in optical density units per 10 µl) against BUN (in milligrams per cent) The numbers in the figure pertain to the cases listed in Table III A maximum of 3 pairs of values for each patient was plotted for the sake of clarity The lettering refers to the first 3 successive samplings for each patient On the vertical line are shown the average and the upper 99 per cent confidence limits of normal urea plasma RNase activity

14 and 19, however, presented moderate increases in RNase activity in spite of a greater concentration of urea in blood and a fatal outcome, whereas Case 10 had a relatively high plasma RNase activity with a low degree of uremia In the group with intermediate levels of RNase activities (Cases 5 to 13), some had

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*It may be seen that dialysis had no effect on plasma RNase activity (Table III Cases 1 and 2)

TABLE III. RIBONUCLEASE ACTIVITY IN BLOOD OF UREMIC PATIENTS WITH BLOOD UREA NITROGEN VALUES GREATER THAN 46.6 MG. PER CENT

CASE NO.	DIAGNOSIS*	PLASMA RNase†	DATE
1	Chronic renal disease; posttraumatic acute renal failure (C)	0.853 1.010 1.046 0.996	4/24 4/28 5/18(a)‡ 5/14(b)‡
2	Renal amyloidosis; acute renal failure post surgery (C)	0.850 0.912 0.520 0.640 0.734 0.751	4/7 4/10 4/18(a)‡ 4/18(b)‡ 4/22 4/23
3	Primary contracted kidney; malignant phase of hypertension (N)	0.820 0.510	4/24 4/26
4	Chronic glomerulonephritis (N)	0.804	5/2
5	Nephrotic syndrome with advanced chronic renal failure (C)	0.610 0.659 0.606 0.619 0.613 0.612	4/10 4/16 4/23 4/28 5/3 5/21
6	Renal tumor with urinary obstruction (C)	0.329 0.526 0.663 0.625	4/14 4/16 4/18 4/22
7	Chronic glomerulonephritis (C)	0.512	
8	Chronic glomerulonephritis (C)	0.452 0.525	5/8 5/22
9	Obstructive uremia, prostatic origin (C)	0.459 0.124	4/24 5/6
10	Chronic kidney failure	0.513	5/17
11	Chronic kidney failure	0.435 0.455	4/24 5/13
12	Chronic glomerulonephritis (N)	0.506	4/23
13	Chronic glomerulonephritis (C)	0.448 0.406	
14	Chronic renal failure; pronephrosis (N)	0.461 0.313 0.326	4/10 4/16 4/18
15	Chronic kidney disease (lupus erythematosus) (C)	0.372 0.354 0.293	4/12 4/18 4/23
16	Postabortum acute renal failure (C)	0.377 0.202 0.231 0.158	4/10 4/14 4/22 5/2
17	Renal rickets (C)	0.277 0.351 0.328 0.274	4/14 4/16 4/18 4/23
18	Acute glomerulonephritis (C)	0.373 0.168 0.199	5/2 5/6 5/8
19	Malignant phase, essential hypertension (N)	0.383	4/18
20	Postoperative vomiting	0.267	4/24
21	Liver cirrhosis (C)	0.235 0.284	4/23 4/24
22	Postoperative vomiting	0.164 0.123 0.113	5/3 5/6 5/12
23	Generalized peritonitis (N)	0.227	5/21

*C = clinical, N = necropsy.

†Optical density per 10 µl of plasma.

‡(a) = After artificial kidney, (b) = before artificial kidney

stationary but severe renal disease (Cases 5, 8, 10, 11, and 13) and others progressed rapidly to death (Cases 6, 7, and 12) Case 6 showed the progressive increase in both urea and RNase with impairment of renal function Case 9 had a favorable course after prostatic obstruction was relieved, with a drop in both urea and RNase activity

DISCUSSION

The above results show that plasma RNase activity increases in the blood of uremic patients Extrarenal uremia does not seem to cause such increases although it is recognized that our data are rather limited Métais and Mandel[4] found no significant variation in serum RNase activity in the blood of 20 patients with several diseases, including patients with renal disease No clinical or laboratory data were given that would permit further analysis of their results Infections have been reported not to influence serum RNase activity[6]

In a previous paper[3] we discussed the hypothesis that a decrease in the glomerular filtration rate could explain the increase in serum RNase activity found after temporary ligation of the renal pedicles in rats A similar hypothesis might account for the increase in serum RNase activity found in uremic patients However, more data are needed on the rate of production and release of the enzyme into the blood, and its mechanism of excretion in the urine[7, 3] before such an hypothesis may be considered to be securely grounded

The increase in RNase activity does not seem to be a consequence of uremia Aside from the behavior of plasma RNase in the cases of extrarenal uremia reported here, this is borne out by experiments in dogs, in which ureterovenous anastomosis was performed, under these conditions, small variations in serum RNase were found, in spite of a NPN retention similar to that of binephrectomized animals[8]

Much more experience with the method on more extensive clinical material is necessary before its possibilities as a diagnostic or prognostic tool may be evaluated *

SUMMARY

Plasma RNase was estimated in blood samples of human subjects containing normal and high concentrations of urea Marked increases in the plasma RNase were found coincident with a high urea concentration in patients with kidney impairment No strict correlation was found, however, in different patients, between blood urea levels and plasma RNase activities

The advice and facilities provided by Drs J B Magaldi, I Nussenzweig, A Cardoso, and W T Beraldo and the technical assistance of Mrs M C Cosentino are gratefully acknowledged The blood urea determinations were performed by the staff of the Central Laboratory, Hospital das Clinicas

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* After this paper was prepared for publication our attention was called to the work of J C Houck and L B Berman (J Appl Physiol 12 473 476 1958) who reported an increase in serum RNase activity in acute renal failure these authors also presented data supporting a correlation between the white blood cell count and serum RNase activity

them by the same water loading protocol as used in the normal subjects. At the end of the control period, the patients with diabetes mellitus were given 30 and 40 units of HGF insulin intravenously.

The one patient with diabetes insipidus was responsive to aqueous Pitressin, and this drug had been withheld for a 3 day period immediately preceding the study. He was not given a water load, and received 0 30 unit HGF insulin per kilogram of body weight after control measurements were obtained.

All normal subjects and the patient with diabetes insipidus received glucose in the maintenance inulin infusion in order to prevent the development of hypoglycemia.

Inulin determinations were performed using Schreiner's modification of the resorcinol method[3] Nelson's adaptation of the Somogyi method was used to determine blood glucose concentrations.[4]

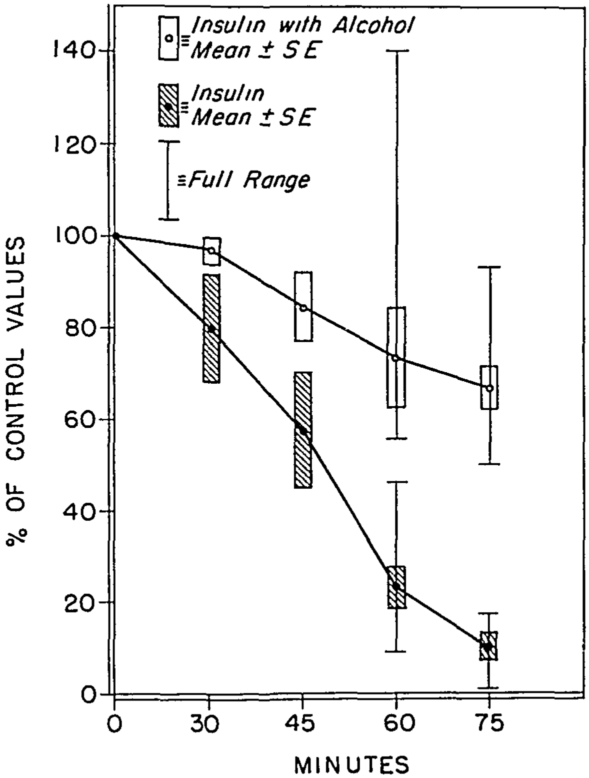

Fig 1—Diagram comparing the rate of urine flow in normal subjects who received insulin and in normal subjects who received insulin and alcohol intravenously The abscissa denotes the time interval following insulin administration Urine flows charted at zero time represent the means of the control values

RESULTS

Control urine flows ranged from 8 to 13 ml per minute in the normal subjects who did not receive alcohol and varied from 9 to 16 ml. per minute in the normal subjects who did receive alcohol Insulin consistently produced an antidiuresis in the normal subjects who did not receive alcohol This antidiuresis was associated with an increase of the urine specific gravity and occurred without a change in glomerular filtration rate Alcohol administered prior to insulin administration partially inhibited the development of antidiuresis (Fig 1) In normal subjects who received only insulin, the rate of urine flow 75 minutes after insulin administration was 0 1 to 1 6 ml per minute In those normal subjects who received both insulin and alcohol, the range of urine flows obtained 75 minutes after insulin administration were 7 3 to 11 ml per minute

After insulin administration in the two patients with diabetes mellitus, antidiuresis was delayed until the disappearance of glucosuria 90 and 120 minutes later None of the other subjects examined had glucosuria at any time during the experiment

Administration of insulin to the patient with diabetes insipidus produced no change in urine flow or urine specific gravity (sp gr = 1 001) His control rates of urine flow were 6 7, 6 2, and 6 8 ml per minute Consecutive 15 minute collections after administration of insulin revealed urine flows of 6 7, 7 2, 7 8, 8 3, and 8 5 ml per minute, respectively

DISCUSSION

The failure of insulin to cause antidiuresis in a subject with diabetes insipidus responsive to Pitressin does not support the concept that insulin produces antidiuresis by a direct effect upon a renal tubular mechanism concerned with water reabsorption Alcohol reportedly inhibits the release of ADH from the posterior pituitary[5] The ability of alcohol to inhibit the antidiuretic response to insulin in normal subjects suggests that the antidiuresis caused by insulin administration is mediated through the action of ADH

The inhibition of insulin antidiuresis by glucosuria (osmotic diuresis) in two subjects with diabetes mellitus is in keeping with this hypothesis

It thus appears that the antidiuresis resulting from the administration of commercially available insulin may be mediated through the release of antidiuretic hormone

SUMMARY

The antidiuresis in response to commercially available insulin is again confirmed The failure of insulin to produce antidiuresis in a patient with diabetes insipidus and the inhibition of insulin antidiuresis in normal subjects by the administration of alcohol suggest that insulin antidiuresis is mediated through the action of ADH, and is not due to a specific effect of insulin upon the renal tubule

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RENAL FACTORS RESPONSIBLE FOR THE HYPERMAGNESEMIA OF RENAL DISEASE

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FEW data are available describing the renal mechanisms of magnesium excretion either in normal man or in patients with renal disease. It is thought that magnesium is excreted in the urine of mammals as the net result of glomerular filtration and tubular reabsorption.[1] In addition, various authors have suspected that, as with potassium, tubular secretion of magnesium may occur.[2-5] Efforts to document the occurrence of tubular secretion have thus far been unsuccessful.[3-6]

It has been recognized for some time that many individuals with kidney disease may demonstrate hypermagnesemia at some point in the course of their disease.[7, 8] It has also been shown that the serum magnesium may be normal or low in such patients.[7] Thus far, no attempts have been made to compare the elevation of total serum magnesium with quantitative measurement of the changes in renal function which occur in patients with various types of renal disease. It was decided to determine whether any relationship existed between the total serum magnesium concentration and the degree of impairment of the glomerular filtration rate in individuals with renal disease.

MATERIALS AND METHODS

Eighteen men between the ages of 34 and 68 were studied. Their filtration rates varied between 9.0 to 93.0 ml. per minute and thus covered a wide range of impaired renal function in patients with kidney disease. Most of the subjects had either chronic pyelonephritis, chronic glomerulonephritis, or benign nephrosclerosis associated with hypertensive vascular disease. All patients received either regular hospital diets or diets that were restricted in sodium and/or protein according to the therapeutic demands of their illnesses. None received either abnormally high intakes of magnesium or any medication known to alter the serum magnesium concentration.

All studies were performed in the forenoon while the subjects were in the fasting, supine state. An adequate urine flow was obtained by the oral ingestion of 800 to 1,000 ml. of water approximately 30 to 60 minutes prior to beginning the study. Urine was collected by an indwelling catheter at intervals of 10 to 20 minutes. The bladder was

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washed with 60 ml. of normal saline and 40 ml. of air at the end of each urine collection period. Venous blood was drawn without stasis through an indwelling No. 18 Cournand arterial type needle into heparinized syringes at approximately the mid-point of each urine collection period. The blood samples were then centrifuged, and the plasma was withdrawn for the determination of inulin and magnesium concentrations.

The filtration rate was measured by the inulin clearance. The inulin was administered at a constant rate by a Bowman constant infusion pump following an initial priming dose. The resorcinol method of Roe and associates as modified by Schreiner[9] was used for the determination of inulin in unyeasted urine and cadmium sulfate filtrates of plasma. A delay time of 2.5 minutes was allowed in the calculation of the inulin clearance.

Total serum magnesium concentration was determined in the trichloroacetic acid filtrate of plasma using *a minor modification of the titan yellow method described by* Garner.[10] The normal range in our laboratory as established by measurements made on 39 normal subjects is 1.7 to 2.2 mEq. per liter (mean 1.93 ± S.D. 0.25 mEq. per liter). No estimation of the amount of protein-bound magnesium was made. Duplicate or triplicate determinations performed on the same sample were required to agree within ± 0.15 mEq. per liter.

Each series of observations on any one patient consisted of at least 4 consecutive urine collection periods.

TABLE I. GLOMERULAR FILTRATION RATE AND TOTAL SERUM MAGNESIUM CONCENTRATION IN PATIENTS WITH RENAL DISEASE

PATIENT	AGE	DIAGNOSIS	GFR (ML./MIN.)	SERUM MAGNESIUM (mEq./L.)
I. T.	63		9	2.98
S. M.*	40		14	2.12*
J. S.	47		16	3.10
C. B.	58		22	2.82
M. P.	64		25	2.62
C. K.	44		27	2.67
H. M.	47	Chronic pyelonephritis	30	2.11
S. Y.	35	Benign nephrosclerosis	41	2.00
F. H.	36	Acute glomerulonephritis	59	1.50
J. W.	34	Acute glomerulonephritis	60	1.64
Z. J.	68	Chronic pyelonephritis, congestive heart failure	63	2.12
C. S.	61	Benign nephrosclerosis	71	2.07
F. A.	63	Chronic pyelonephritis	72	2.05
M. H.	36	Obstructive uropathy	72	1.32
J. K.	38	Benign nephrosclerosis	73	1.80
J. Ki.	41	Benign nephrosclerosis	80	2.10
R. W.	35	Mitral stenosis, azotemia	84	2.17
O. R.	38	Acute glomerulonephritis, subsiding	93	1.91

*Receiving oral chlorothiazide 0.5 to 1.0 Gm. daily.

RESULTS

The results are summarized in tabular form in Table I. All values for filtration rate represent the means of at least 3 consecutive urine collection periods. Similarly, the serum magnesium concentrations represent the means of the determinations performed on the blood samples drawn at approximately the mid-point of each urine collection period. *Without exception, the total* serum magnesium concentration did not exceed the normal range of 1.7 to 2.2 mEq. per liter until the filtration rate was reduced to approximately 30 ml. per minute or below. At that point, with only one exception, the serum magnesium rose to well above the upper limits of normal as determined in our laboratory (2.2 mEq. per liter). The one patient whose serum magnesium remained within normal limits despite a reduction in filtration rate below 30 ml. per minute had been receiving daily doses of chlorothiazide prior to the study.

DISCUSSION

Renal mechanisms for the excretion of magnesium must play an important role in regulating the plasma magnesium level. It can be seen from the above data that the occurrence of hypermagnesemia in renal disease is closely related to the degree of reduction in filtration rate. When the filtration rate was reduced to (or below) 30 ml. per minute, all values for serum magnesium were consistently elevated with but one exception. Similar observations have been made in relation to the rise of serum phosphorus which occurs in renal failure.[11] Recently, Hammarsten and Smith[12] reported a positive correlation between the rise of serum magnesium and the increase in nonprotein nitrogen which occurs in patients with renal disease. Their data, as well as ours, would support the concept that one of the most important variables determining the renal excretion of magnesium is the glomerular filtration rate. In normal man, probably not more than 8 to 10 per cent of the filtered load of ionized magnesium is excreted in the urine per 24 hours. With a reduction in the filtered load of magnesium as in renal disease, less magnesium is available for excretion in the urine, and the serum concentration rises. This would be particularly true if either the tubular reabsorptive mechanisms remained intact or if passive back-diffusion occurred through damaged tubule cells. Since no partition of the total serum magnesium was done, it is impossible to state whether or not the increase of serum magnesium is primarily of the ionized component. Other data indicate that such is the case in uremia.[13] Excluding the possibility of the filtration of protein-bound magnesium through damaged glomeruli, this would seem a reasonable observation since a reduction in filtration rate is closely related to the rise of serum magnesium. The present data certainly provide no information as to whether or not tubular secretion of magnesium may occur, but would indicate that if such a mechanism does exist that it is quantitatively unable to maintain a normal serum, magnesium level in the presence of a fall in filtration rate to 30 ml. per minute.

Our observations also provide an explanation for the conflicting data in the literature as to whether the total serum concentration of magnesium is high, normal, or low, in patients with renal disease. Since quantitative changes in renal function studies had not been related to the serum magnesium concentration in these previously reported cases, it seems probable that the normal or low serum magnesium values were found in patients with filtration rates higher than 30 ml. per minute.

Under certain conditions, the serum magnesium may be normal despite a reduction in filtration rate to 30 ml. per minute or below. Our patient (S. M.) had been receiving 0.5 to 1.0 Gm. of oral chlorothiazide daily for 8 to 10 days prior to the study period There is some evidence that chlorothiazide may increase the urinary excretion of magnesium in normal man[14] Such a drug action could be responsible for the normal serum magnesium in this patient Unfortunately, careful studies as to the effect of chlorothiazide on the urinary excretion and serum concentration of magnesium were not feasible in this patient Other drug and/or dietary factors which affect the serum level of magnesium must be considered in addition to the possible effects of chlorothiazide It has been previously shown that the intravenous infusion of calcium salts in dogs results in an increase in the urinary excretion of magnesium[1] Other data suggest that perhaps the fall of serum magnesium in patients with normal renal function following the intravenous administration of calcium gluconate is due to the movement of extracellular magnesium into the intracellular compartment or bone, and not due to an increase in the urinary excretion of magnesium[15] It does not seem likely that patients with filtration rates of 30 ml per minute, or lower, would ingest a diet containing such small amounts of magnesium as to result in depression of the serum value On the other hand, conditions of magnesium loading might raise the serum level in patients with filtration rates higher than 30 ml per minute Excluding such drug or dietary factors, a rise in the total serum magnesium concentration in subjects with renal disease is related to the magnitude of fall in filtration rate

SUMMARY

1 Simultaneous determinations of filtration rate and total serum magnesium concentration were done on 18 men with various types of renal disease

2 With one exception, all patients with filtration rates less than 30 ml per minute exhibited a distinct rise in the total serum concentration of magnesium above normal values

3 One patient receiving chlorothiazide had a normal serum magnesium level in the presence of a fall in filtration rate to below 30 ml per minute

4 The hypermagnesemia of renal disease is closely related to the degree of reduction in glomerular filtration rate

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STIMULATION OF GASTRIC PEPSIN SECRETION IN MAN BY HYPERVENTILATION AND BY DIAMOX

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IN ATTEMPTING to interfere with the carbonic anhydrase mechanism of gastric secretion[1, 2] in man, the effects of hyperventilation, to reduce the CO_2 supply, were contrasted to the effects of inhibition of the enzyme itself by Diamox. Both hyperventilation[7] and Diamox[3, 4] have been shown previously to reduce the secretion of hydrogen ions, but none of the reported studies have described any measurements of the effects on other constituents of gastric secretions. This study reports the effects of hyperventilation and of Diamox on H, Cl, Na, K, pepsin, and protein in the gastric juice. Both caused only modest decreases in acid secretion, but produced highly significant increases in pepsin secretion. The report deals with attempts, so far unsuccessful, to identify the mechanism of stimulation of pepsin secretion by either hyperventilation or by Diamox.

METHODS

Studies were performed on 14 fasting subjects, comprising 7 normal students, 2 vagotomized men with otherwise intact stomachs, and 5 patients with peptic ulceration (2 gastric and 3 duodenal). Experiments were done either in the basal state or during the intravenous infusion of 400 μg histamine base per hour, given as described elsewhere.[6] After a reasonably constant rate of secretion was found for 3 consecutive 15 minute periods, voluntary hyperventilation was performed for 15 or 30 minutes in 20 experiments. All subjects had tingling of the extremities at least and most exhibited Chvostek's sign; several also had carpopedal spasm.

Diamox (2 acetylamino 1, 3, 4 thiadiazole 5 sulfonamide) was injected intravenously in 5 subjects in doses of from 20 to 75 mg. per kilogram of body weight in 15 to 30 minutes; 3 were injected during histamine infusion and 2 while in the basal state.

Gastric juice was collected through a Levin tube by constant suction, supplemented at 15 minute intervals by hand suction with a syringe to complete consecutive 15 minute samples; these were assayed for free H^+ ion (end point pH 3.3), total chloride concentration, and pepsin concentration by methods previously described,[6] as well as for Na^+ and K^+ concentration by flame photometry; protein concentrations were measured with the biuret reagent. Forearm venous blood was collected without stasis in heparinized syringes and the pH measured immediately in 17 hyperventilation experiments.

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RESULTS

Hyperventilation.—

During histamine infusion: The effects of hyperventilation on the steady-state maximal secretion during histamine infusion was studied in 9 experiments on 7 subjects (Table I). Fifteen minutes of hyperventilation produced a highly significant prompt increase ($p < 0.005$) in concentration and output of pepsin and protein in the gastric juice, and a modest, although probably significant ($p < 0.05$) drop in H ion concentration. The output of Na^+ increased but this was probably not statistically significant ($p = 0.05$). These effects persisted for 15 minutes after the end of the hyperventilation. No significant effects on volume, on chloride, or potassium were observed. Volume increased in 2 or 3 subjects, but identical changes in composition of the juice occurred and were as marked in the others who had no changes in volume secretion.

TABLE I. EFFECT OF HYPERVENTILATION FOR 15 MINUTES DURING CONTINUOUS HISTAMINE INFUSION ON VOLUME AND COMPOSITION OF GASTRIC JUICE IN 9 STUDIES (MEANS ± S.E.M.)

PERIOD	MINUTES	VOLUME (ML./ 15 MIN.)	P.U./ML.	PROTEIN (MG. %)	H^+ (mEq./L.)	Na^+ (mEq./L.)	K^+ (mEq./L.)	Cl^- (mEq./L.)
Control	−30	57.4 ± 9.9	1,410 ± 201	310 ± 25	117 ± 5.9	13 ± 2.3	18.1 ± 0.6	156 ± 3.3
	−15	50.0 ± 5.0	1,223 ± 135	310 ± 40	127 ± 5.0	12 ± 2.4	18.0 ±0.7	162 ± 4.4
Hyperventilation	15	66.2 ± 9.3	2,162* ± 240	534† ± 48	112 ± 4.5‡	16 ± 1.9	18 ± 0.9	155 ± 3.6
Recovery	30	56 ± 5.6	1,765‡ ± 204	444‡ ± 63	113 ± 6.3‡	16 ± 2.1	17.5 ± 0.9	156 ± 4.0
	45	55 ± 6.2	1,390 ± 171	398 ± 74	121 ± 3.7	15 ± 2.2	16.8 ± 0.7	157 ± 3.6

P.U. = pepsin units.
*$P<0.005$; by "t" test significance of difference of mean from mean control −15 minutes.
†$P<0.001$.
‡$P<0.05$.

TABLE II. EFFECT OF 30-MINUTE HYPERVENTILATION DURING BASAL SECRETION ON VOLUME AND COMPOSITION OF GASTRIC JUICE IN 2 SUBJECTS (MEAN VALUES)

PERIOD	MINUTES	VOLUME (ML./ 15 MIN.)	P.U./ML.	PROTEIN (MG. %)	H^+ (mEq./L.)	Na^+ (mEq./L.)	K^+ (mEq./L.)	Cl^- (mEq./L.)
Control	−30	28	1,150	750	39	58	15.0	122
	−15	28	1,080	750	43	58	14.0	130
Hyperventilation	15	45	2,255	620	45	62	14.0	129
	30	30	2,560	1,030	62	44	15.5	135
Recovery	45	46	1,675	880	68	44	14.8	138
	60	26	1,480	525	81	31	14.4	143

In the basal state (Table II): In 2 subjects, hyperventilation for 30 minutes produced the same striking pepsin increases during the first 15 minutes in the basal state as during histamine infusion. The H^+ concentration, however, did not fall and in fact started rising during the second 15-minute period and continued to increase after that, accompanied by an increase in chloride concentration and a concomitant fall in sodium concentration. Potassium was unaffected. The stimulation of pepsin secretion was therefore quicker and out of proportion to the stimulation of acid secretion.

Thirty-minute Hyperventilation.—Two successive 15-minute periods of hyperventilation were performed by 5 subjects, 3 during histamine and 2 in the basal state. In one basal and one histamine study, the plateau of pepsin concentration was reached in the first period; in the other 3, the pepsin concentration continued to rise in the second period and in 3 subjects reached levels not seen even in hypoglycemic or any other type of stimulation. For the 5 subjects the mean concentrations for 6 successive 15-minute periods (2 control, 2 hyperventilation, and 2 after) were 1,630, 1,400, *2,682*, *3,250*, 2,062, and 1,572 pepsin units per milliliter, respectively.

Other experiments were done to investigate possible mechanisms whereby hyperventilation might produce these effects on pepsin and protein secretion.

Cholinergic Pathways.—

Central cholinergic (vagal): Two patients, who had been vagotomized 1 and 5 years before, respectively, and who had intact stomachs were studied. The completeness of vagal section in both was confirmed by insulin hypoglycemia, which in neither produced any stimulation of gastric secretion within an hour of adequate hypoglycemia (true blood glucose less than 40 mg. per cent).

TABLE III. EFFECT OF 15-MINUTE HYPERVENTILATION DURING HISTAMINE INFUSION IN VAGOTOMIZED SUBJECTS (MEAN OF 3 EXPERIMENTS)
(NOTE: DELAYED INCREASE IN PEPSIN CONCENTRATION)

PERIOD	MINUTES	VOLUME (ML./15 MIN.)	P.U./ML.	PROTEIN (MG. %)	H^+ (mEq./L.)	Na^+ (mEq./L.)	K^+ (mEq./L.)	Cl^- (mEq./L.)
Control	–30	22	1,570	–	62	36	16.7	123
	–15	20	1,432	–	62	30	16.7	115
Hyperventilation	15	31	1,667	–	67	35	14.1	123
Recovery	30	21	2,187	–	64	44	13.3	128
	45	28	1,350	–	67	42	12.5	125

Three hyperventilation studies were carried out during subsequent histamine infusion (Table III) and in both subjects a well-marked increase in pepsin secretion was noted. The response differed somewhat from the normal subjects in that the acid concentration did not fall and the maximal concentrations of pepsin were found in the 15-minute period after hyperventilation. In no instance was this the case in the intact subjects where the maximal increases always occurred during the hyperventilation.

Peripheral cholinergic mechanism: In 3 subjects with intact stomachs a dose of atropine was determined which would prevent hypoglycemic stimulation of gastric secretion (between 1.0 and 1.5 mg. of atropine sulfate intravenously). These doses of atropine were given during subsequent histamine infusions and as will be seen by comparing Tables I and IV, atropine under these conditions decreased volume as well as the pepsin, protein, and potassium concentrations. These effects of atropine last for at least an hour. Hyperventilation during these 3 studies produced a fall in H^+ concentration and a rise in sodium, pepsin, and protein concentrations (Table IV) as was the case without atropine (Table I). The increase in pepsin and protein persisted at a high level in the postventilation period, thus resembling the response in the vagotomized subjects.

TABLE IV. EFFECT OF 15-MINUTE HYPERVENTILATION OF 3 ATROPINIZED SUBJECTS DURING HISTAMINE INFUSION (MEANS)

PERIOD	MINUTES	VOLUME (ML./15 MIN.)	P.U./ML.	PROTEIN (MG. %)	H^+ (mEq./L.)	Na^+ (mEq./L.)	K^+ (mEq./L.)	Cl^- (mEq./L.)
Control	–30	49	780	195	123	16.7	12.7	155
	–15	29	630	190	125	15.3	12.0	152
Hyperventilation	15	36	1,373	410	109	21.3	11.8	150
Recovery	30	42	1,307	390	104	31.3	12.8	153

Noncholinergic Mechanisms.—

Possible deionization of calcium: The possibility of preventing the effects of hyperventilation by concomitant calcium administration was studied in two subjects, who were hyperventilated on 3 occasions during histamine infusion and were found to give the typical normal response as seen in Table I. During a subsequent period of hyperventilation in the same study, 30 ml. of 10 per cent calcium gluconate was injected intravenously. One subject had tetany during hyperventilation whether calcium was injected or not; the other had tetany without calcium but no tetany with calcium. Pepsin secretion increased to an extent not significantly different from that seen without calcium injections.

Changes in Blood pH.—

By hyperventilation: Following hyperventilation the pH of the blood rose by 0.07 to 0.30 pH (mean 0.156) unit in all experiments except in one, where the level remained unchanged at pH 7.68. There was no correlation between blood pH and the concentration of pepsin in the gastric juice ($r = +0.26$) (Fig. 1), both apparently increasing independently of the other during hyperventilation. It will be seen that even before hyperventilation the blood pH was above normal, the result of the removal of secreted gastric acid. The increase in pH with hyperventilation was not correlated with the fall in H+ concentration which occurred.

By infusion of bicarbonate (Table V): In 3 of the subjects in whom hyperventilation had produced typical changes in gastric juice composition, an attempt was made to reproduce the alkalosis by rapidly infusing intravenously 5.6 per cent sodium bicarbonate, during established I.V. histamine stimulation. The 3 subjects received 180, 200 and 350 ml of this solution (119, 133 and 233 mEq of base), respectively in 15 minutes. In each the blood pH rose by 0.1 pH unit or more, and the first had tetany. None of the effects of hyperventilation on gastric secretion were reproduced by the bicarbonate alkalosis. A significant ($p < 0.005$) progressive drop in potassium concentration in all 3 subjects was the only gastric effect noted.

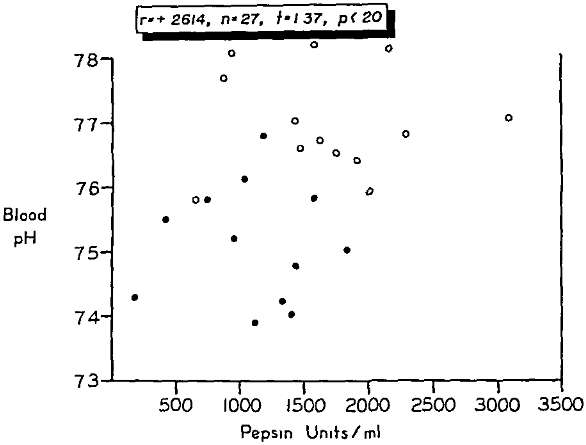

Fig. 1.—Blood pH and pepsin output before (●) and after (○) hyperventilation showing lack of correlation.

TABLE V. EFFECT OF INTRAVENOUS INFUSION OF 5.6 PER CENT $NaHCO_3$ DURING CONTINUOUS HISTAMINE INFUSION IN 3 SUBJECTS

PERIOD	MINUTES	VOLUME (ML/15 MIN.)	P.U./ML	PROTEIN (MG %)	H (mEq/L.)	Na (mEq/L.)	K (mEq/L.)	Cl (mEq/L.)
Control	−45	68	1 2[illegible]0	3[illegible]0	122	16	19.3	158
	−30	78	1 603	450	125	14	20.0	160
	−15	68	1 270	330	116	20	17.7	158
Bicarbonate Infusion in 15 Minutes								
	15	56	1 340	260	113	21	16.6	152
	30	65	1 523	320	123	21	15.3	154
	45	60	1 510	265	126	15	16.0	157
	60	48	1 370	320	132	11	15.7	15[illegible]

Plasma Pepsinogen.—As an incidental observation in 2 subjects in whom an adequate pepsin protein response to hyperventilation was observed no change occurred in the concentration of pepsinogen in the plasma. It was not measured in the other subjects. This suggests that the peptic cell does not develop a nonspecific increase in permeability to protein as a result of hyperventilation.

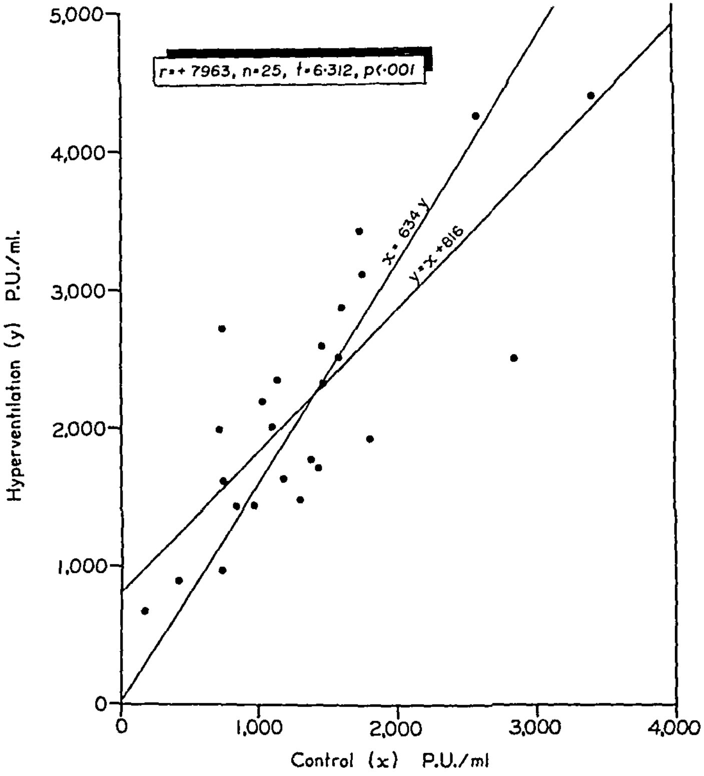

Fig. 2.

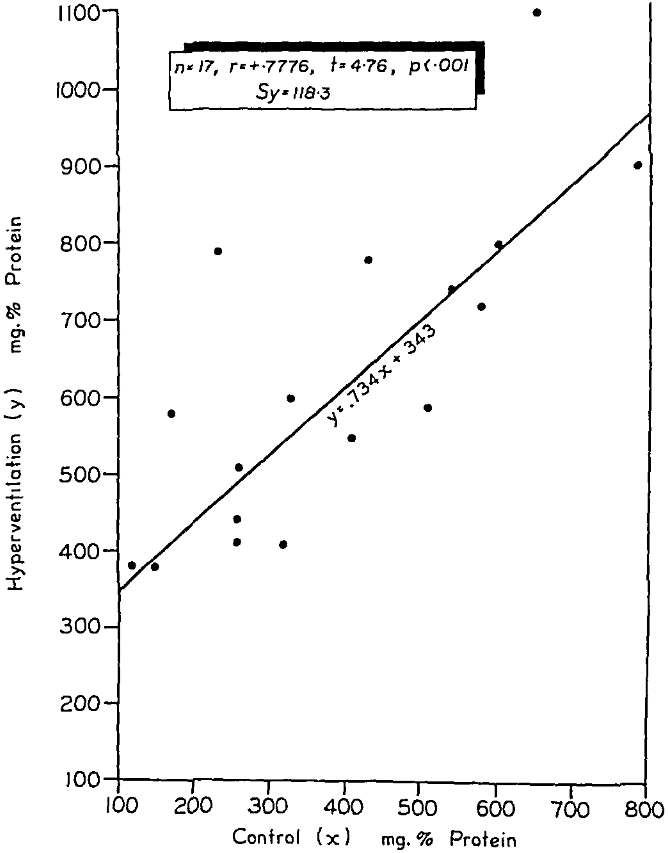

Fig. 3.

Fig. 2.—Significant positive linear correlation between gastric juice pepsin concentrations before and during hyperventilation.
Fig 3.—Positive linear correlation between gastric juice protein concentrations before and during hyperventilation.

The nature of the pepsin increase Fig 2 shows the concentration of pepsin during hyperventilation in all the studies, plotted against the concentration in the preceding 15 minute period It includes the five 30 minute studie in which the pepsin concentration in the second 15 minute period is plotte against that in the first The correlation is positive, linear, and highly significant (r = + 0.796, p < 0.001) A similar significant (r = + 0.792) correlatio was found between the *output* of pepsin before and during hyperventilation In 17 studies where the protein concentration was measured, a similar positiv linear correlation was found (Fig 3) (r = + 0.776, p < 0.001)

The increase in protein is probably accounted for by the increase in pepsin concentration, as suggested by the mean concentration of pepsin per mill gram of protein These are 4.54, 3.95 (hyperventilation), 4.04, 3.98, and 3.4 protein units per milligram for the respective 15 minute periods in the histamine studies In the individual cases, however, the ratio of pepsin to protei is not as constant as it is for the whole group No attempt has been made t determine more exactly the specific potential peptic activity of the protein i the gastric juice

TABLE VI EFFECTS OF DIAMOX DURING CONTINUOUS HISTAMINE INFUSION ON GASTRIC JUIC
VOLUME AND COMPOSITION (MEANS ± S E M)

PERIOD	VOLUME (ML / 30 MIN)	P U /ML	H (mEq /L)	Na⁺ (mEq /L)	K (mEq /L)	Cl (mEq /L
Mean 2 hour control (n = 20)	81.3 ± 11.8	1,132 ± 74	75.9 ± 7.3	24.0 ± 2.4	16.57 ± 0.34	127 ± 7
			Diamox Intravenous			
Post Diamox 30 minutes (n = 10)*	82.2 ± 19.9	1,233 ± 82	80.3 ± 11.7	24.2 ± 2.0	14.2 ± 0.48	126 ± 10
Post Diamox 60 minutes	65.6 ± 11.6	1,477 ± 87	72.5 ± 10.3	31.1 ± 2.7	13.0 ± 0.49	125 ± 9
Post Diamox 90 minutes	64.8 ± 15.1	1,552 ± 128	55.9 ± 11.8	41.3 ± 3.5	11.8 ± 0.42	128 ± 7
Post Diamox 120 minutes	66.0 ± 14.1	1,667 ± 109	63.3 ± 12.6	44.1 ± 7.2	11.3 ± 0.95	128 ± 6

*n = Number of samples

The Effects of Diamox—Diamox in a total dosage of 1.2 to 3.75 Gm (20 t 75 mg per kilogram of body weight) was given intravenously to 3 subjects during histamine infusion and to 2 others during basal secretion The effec were essentially similar and are combined in Table VI The reduction in bot volume and in acid concentration each not quite significant statisticall resulted in a marked reduction in the calculated output of acid following th injection of Diamox The chloride concentration however remained unchanged, the fall in potassium concentration was highly significant as w the rise in sodium concentration The sum of cations like the concentratio of chloride remains unchanged after Diamox The concentration of pepsi increased significantly and continued to rise steadily up to 2 to 3 hours afte the Diamox injection

DISCUSSION

With the present state of knowledge of pepsinogen secretion[10] it is no possible to interpret the gastric juice pepsin increases following hyperventilation or Diamox in terms of mechanisms

It would seem probably that hyperventilation exerts its effect on pepsin secretion neither through the vagus nor through local cholinergic mechanisms, and, furthermore, that the effects are probably not due to an alteration of blood pH per se nor to altered cell permeability to pepsinogen. The role of calcium deionization has not been fully evaluated if, in fact, such deionization occurs with hyperventilation.[8] The possible role of increased gastric motility, which occurs after hyperventilation,[9] has not been evaluated, but it is difficult to see how such changes in motility could be concerned with an increase in pepsin secretion. Furthermore, the persistence of increased pepsin and protein secretion suggests that the mechanical act of hyperventilation as such has no direct effect on pepsin secretion. Whatever the mechanisms, however, they appear to act independently of the known pathways of stimulation of pepsin secretion in man, where both acid and pepsin secretion are simultaneously stimulated[10] by the known stimulants.

The changes in hydrogen ion which occurred with both hyperventilation and with Diamox might be interpreted in terms of the carbonic anhydrase mechanisms thought to be responsible for the supply of hydrogen ions to the secreting mucosa. These occurred without significant changes in chloride concentrations, and are similar to the dissociation of H and Cl which occurs when an electric current is passed from mucosa to serosa, opposite in direction to the normal mucosal electromotive force.[7]

SUMMARY

In 25 studies on 14 subjects voluntary hyperventilation for 15 to 30 minutes invariably caused a very significant increase in the concentration and output of pepsin in the gastric juice. This occurred whether hyperventilation was performed during continuous histamine infusion, in the basal state, following complete vagotomy, or during adequate atropinization. It could not be reproduced by equivalent alkalosis produced by intravenous $NaHCO_3$ infusion. The effect persisted for 15 minutes after the cessation of hyperventilation. The mechanisms of this response have not been elucidated and appear to involve none of the known pathways for stimulation of pepsin secretion. Diamox given intravenously also increased the secretion of pepsin.

The effects of hyperventilation and of Diamox on gastric juice volume and hydrogen ion, sodium, potassium, chloride, and protein concentrations reported in the paper shed no further light on the mechanisms of pepsin stimulation.

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COMPARATIVE TOLERANCE TO POISONS OF NORMAL LIVER TISSUE AND REGENERATED LIVER TISSUE AFTER MAJOR HEPATECTOMY

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After the surgical removal of 70 per cent or more of the liver in human beings and dogs, sustenance of life is dependent on the ability of the normal residual remnant plus its regenerated component to carry out the vital functions commonly attributed to this organ. Its ability to do so has been proved by clinical experience and experimental laboratory investigations. The entire right lobe of the human liver is being resected with increasing frequency for such indications as primary malignant tumors (hepatoma, cholangiocarcinoma, and somatic sarcoma, unilobar metastases, cancer of the gall bladder, and occasional benign tumors. The right lobe consists of 80 per cent of the weight of the human liver, yet it now is feasible and relatively safe to perform total right hepatic lobectomy by the controlled hemostatic procedure of preliminary ligation and severance of the right hepatic duct, the right hepatic artery, and the right branch of the portal vein prior to *transection of the interlobar septum.* A residual 20 per cent of the liver substance is enough for the maintenance of life if the subject is an otherwise normally functioning organism. The tested liver functions are minimally disturbed by the removal of the human right hepatic lobe except for transient hypoproteinemia.

In our experiments, the rapidity and completeness of liver regeneration after subtotal hepatectomy appear to be inversely proportional to the size of the animal. In rats, the liver has completely regenerated within 12 to 18 days after major lobectomies. In dogs the remnant of residual liver after 70 per cent hepatectomy has grown to the original proportion within 8 weeks after the operation. In the human being the process of regenerative hyperplasia is not so easily followed because of the infrequency of "second-look" observations and measurements, but we found in two patients, on exploratory

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laparotomy 2 years after total right hepatic lobectomy, that the left lobe had expanded to fill the entire cupola of the diaphragm, assuming final dimensions as great, or greater, than the original liver.

The minimal deviations from normal in the numerous functions of the liver after total right hepatic lobectomy have been a source of security for the patient and of assurance to the surgeon. Not so much is known of the regenerated liver's tolerance or adaptation to unusual or "stress" conditions to which even the normal intact liver is not ordinarily subjected. One such function is the detoxification of poisons. We thought it of interest to see if the regenerated liver of dogs possessed the ability to detoxicate carbon tetrachloride to the same degree as the normal intact liver of other dogs.

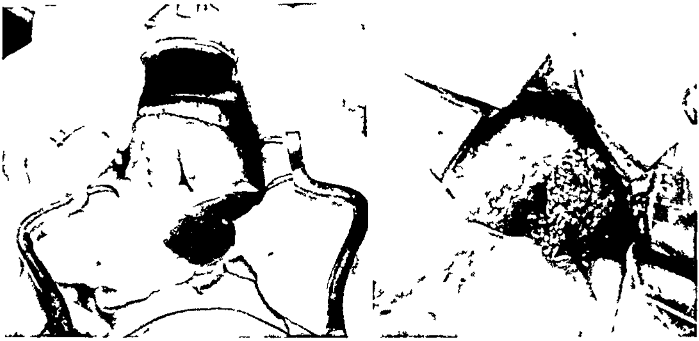

Fig 1.—*A*, Experimental cirrhosis in dog with intact liver. View at laparotomy of an adult mongrel dog which received 70 ml of carbon tetrachloride over a period of 9 months. The liver appears coarsely nodular with a uniform but distributed mottled appearance involving the entire capsular surface. The nodules range in size downward from 1 cm in diameter. The liver is pale in contrast to the deep red spleen. *B*, Experimental cirrhosis in dog after major hepatectomy. View at laparotomy of the liver of a dog which received carbon tetrachloride during a 9 months course. The exhibition of the drug was initiated 4 months after a 70 per cent partial hepatectomy. The 70 per cent partial hepatectomy was performed 2 months after a previous 50 per cent hepatectomy. The liver surface was nodular and tan with some patches of reddish hue.

METHODS

Carbon tetrachloride was used as the toxic agent. Dogs of equal weight were used for this study. Some dogs had been operated on two months before the experiment started and had 70 per cent of their livers removed. The control dogs had no operation and therefore their livers were intact. At the beginning of the experiment these dogs were weighed and carbon tetrachloride was inserted through an isolated loop of ileum. Three milliliters of carbon tetrachloride was introduced every other day for 3 months. Then enteric capsules containing 5 ml of carbon tetrachloride were given by mouth to these dogs every other day for 6 months. All dogs were weighed weekly and liver function tests were done each month. No special diet was given. At the end of the experiment all dogs were surgically explored. The peritoneal cavity contained 600 to 1,200 ml of clear, yellowish fluid. The livers of both groups of animals were granular, hard, and yellowish. Small segments of these cirrhotic livers were removed for histologic study.

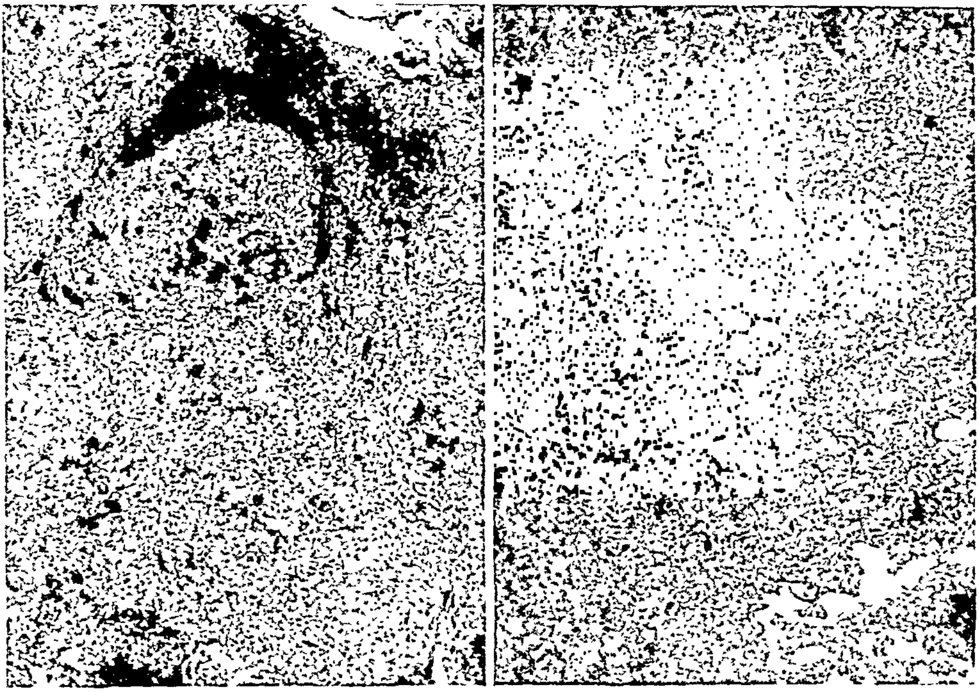
A. B.

Fig. 2.—*A*, Liver of an adult mongrel dog which had received 270 ml. of carbon tetrachloride over a period of 9 months, starting 4 months after a 70 per cent hepatectomy. The 70 per cent partial hepatectomy was performed 2 months after a 50 per cent partial hepatectomy. This low-power microphotograph shows extensive fresh hemorrhage in regions of necrosis. Significant remnants of residual normal hepatic architecture cannot be identified in this section. The sinusoidal patterns are absent. *B*, Same liver as in *A*. The liver in this biopsy shows acute massive necrosis. In the center of the microphotograph are intact surviving parenchymal cells, surrounding vascular channels. There are foci of fresh hemorrhage infiltrating the necrotic parenchyma. The sinusoidal channels are not patent.

RESULTS

1. The alterations of liver function tests in those dogs with regenerated livers and the other dogs without partial hepatectomy were essentially the same.

2. The transient hypoproteinemia (hypoalbuminemia and hypoglobulinemia) associated with the toxic necrosis was considerably less and was restored earlier in the dogs with regenerative hyperplastic livers following major hepatectomy than in the control dogs with intact livers.

3. The weight losses in both groups were somewhat similar, but 7 months from the date the experiment started both groups began to gain weight, which was due to ascites as a result of hepatic cirrhosis.

4. In the dogs which had no partial hepatectomy prior to the administration of carbon tetrachloride, more fluid was noted in the peritoneal cavity and the liver was firmer and more granular (Fig. 1, *A* and *B*). The histologic evidence of the toxic necrosis was of apparently equal degree in both groups of animals. (See Fig. 2, *A*, *B*, *C*, and *D*.)

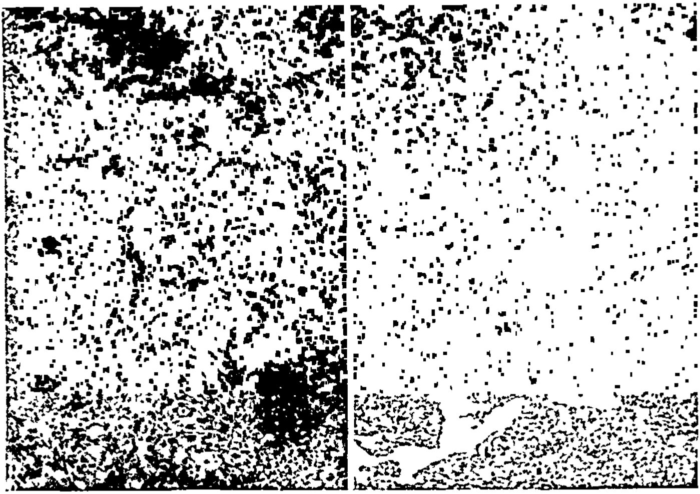

Fig. 2—Cont'd. *C*, Biopsy of same liver as in *A* and *B*. Intermediate magnification The residual viable parenchymal cells show no evidence of glycogen and normal sinusoidal patterns Two large foci of hemorrhage into regions of necrosis *D*, Biopsy of same liver as in *A*, *B*, and *C* Extensive necrosis seen in the lower half of the microscopic field The parenchymal cells are not oriented in the normal array of plates There is no visible evidence of intracellular glycogen storage

CONCLUSION

According to our investigation, the regenerated liver of the dog tolerates poison (specifically, carbon tetrachloride) as well as normal, intact liver, if not more so. The reason for this relative tolerance may be the fact that 2 months or more after major hepatectomy in the dog, the regenerated liver is larger and weighs more than the original liver. Its size represents true regenerative hyperplasia as we have shown by (1) chemical analyses for lipids and nitrogen of aliquot parts of the liver, (2) nuclear counts, and (3) comparable histologic structure as witnessed on microscopic study.

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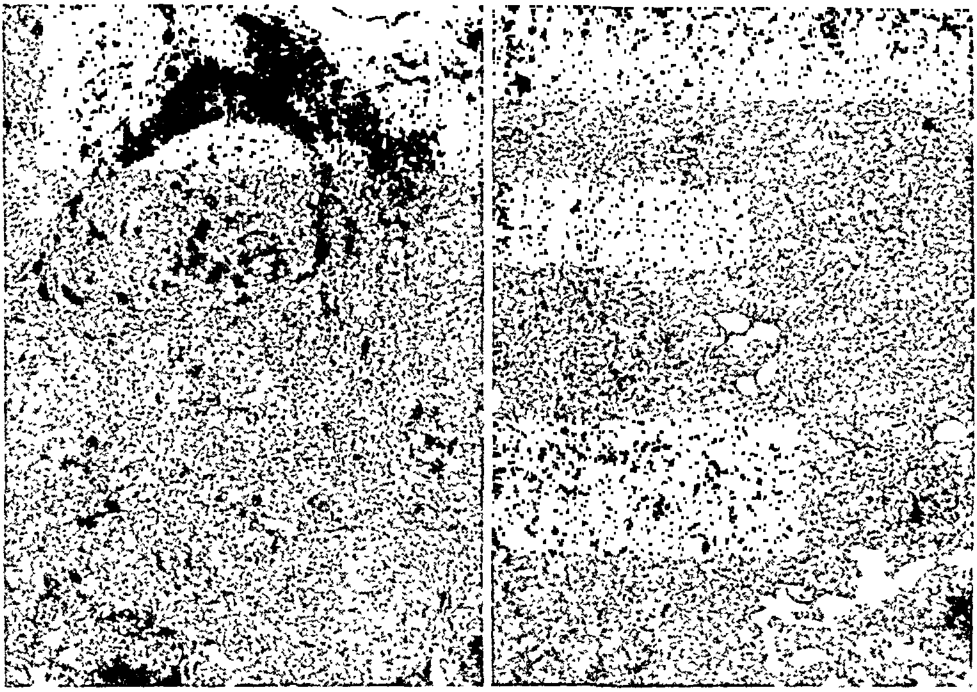

A. *B.*

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LETHAL IRON OVERLOAD IN DOGS

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The harmful effects of large tissue iron deposits have received much attention in recent years In a previous study from this laboratory,[1] dogs which had received intravenously 0 5 to 1 0 Gm of iron per kilogram of body weight lived for 4 to 7 years without developing the characteristic lesions of hemochromatosis in man The purpose of this paper is to describe the effects in dogs of iron injected parenterally in regular doses until toxic manifestations appeared The animals were then observed until death occurred or seemed imminent and the tissue changes at autopsy were recorded One dog received saccharated iron oxide intravenously, 2 received iron dextran intramuscularly, and 2 received iron dextran intraperitoneally The accumulation of 2 5 to 3 3 Gm of iron per kilogram of body weight produced death within 5 to 10 months The tissues showed massive hemosiderin deposits—chiefly in cells of the reticuloendothelial system—with no fibrous tissue reaction All the animals showed anorexia, weight loss, and a decrease in serum albumin concentration, one developed ascites, anasarca, and chronic leg ulcers None of the dogs showed any evidence of diabetes or cirrhosis by laboratory or postmortem examinations The exact cause of death and its relationship to the iron overload were not established

MATERIAL AND METHODS

Two adult mongrel dogs were given injections of the iron dextran solution (Imferon†) intramuscularly into alternate thighs in a dose of 2½ or 5 ml (125 or 250 mg elemental iron) per day 6 days a week The schedule and duration of injections are shown in Fig 1 Dog 1 received a total of 23 Gm of iron (3 1 Gm /Kg), and Dog 2 received 25 Gm (2 5 Gm /Kg) during a 5 month period

Two littermate puppies (approximately 4 months old) were given injections of Imferon intraperitoneally into the left lower quadrant of the abdomen The dosage schedule was 125 mg of iron twice weekly for 3 weeks, then 250 mg twice weekly for 19 weeks, and finally 250 mg daily for 6 days in one puppy (Dog 4) and 500 mg daily for 12 days in the other puppy (Dog 3) The total amount of iron injected was 9 75 Gm (3 3 Gm /Kg) and 14 Gm (2 5 Gm /Kg), respectively (Fig 2)

Another adult mongrel dog was given injections of saccharated iron oxide (Ferrivenin†) intravenously in doses of 10 ml (200 mg of iron) per day 5 days a week for 28 weeks A total dose of 29 Gm of iron (3 0 Gm /Kg) was given to this dog

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The animals were housed in individual cages and were fed commercial dog chow. Hexylresorcinol as a vermifuge was given to each animal during an initial period of observation. Feces were tested for occult blood frequently, and the vermifuge treatment was repeated whenever blood was detected. The dogs were weighed at frequent intervals.

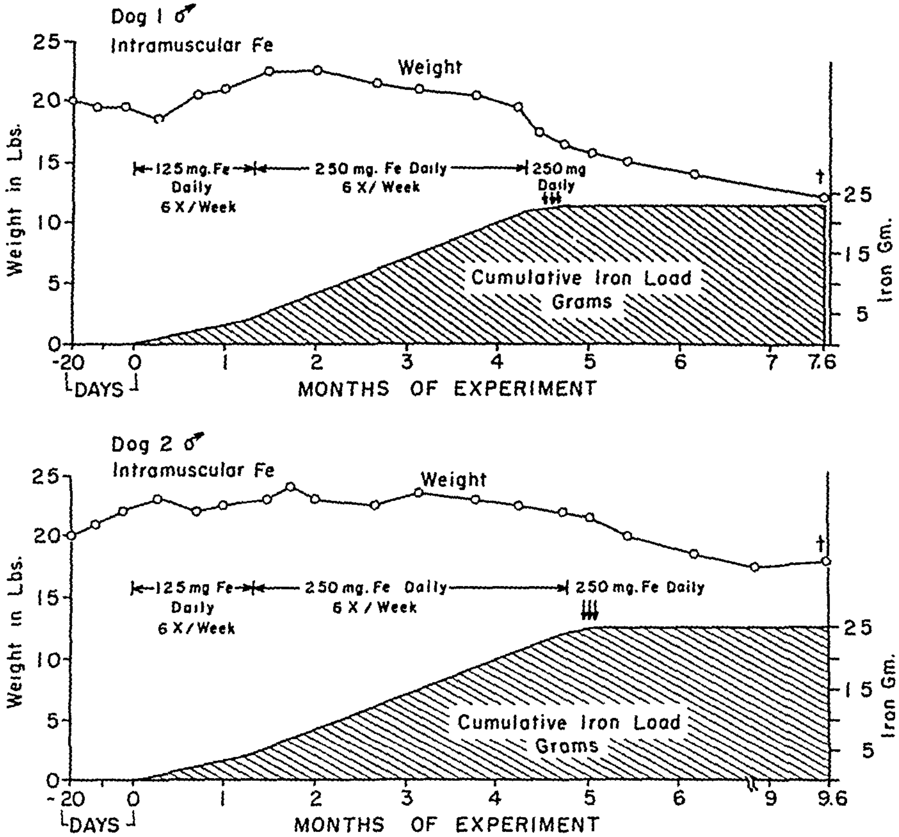


Fig. 1.—Correlation of body weight with cumulative iron load in dogs receiving intramuscular iron-dextran injections.

Periodic determinations of red cell counts, hemoglobin concentration, packed cell volumes, white cell counts, reticulocytes, serum iron, and unsaturated iron binding capacity were made by methods previously described.[1] In addition, measurements of fasting blood sugar, serum bilirubin, Bromsulphalein retention and serum protein fractions by chemical and paper electrophoretic techniques were performed at intervals on the dogs which received iron-dextran. At death, tissue iron estimations were made as previously described.[1] Histologic examination was performed on tissues fixed in neutral formalin and Zenker's acetic solutions. Sections were stained routinely with hematoxylin and eosin and for iron with a modified Gomori stain counterstained with Mallory's basic fuchsin.

RESULTS

A. Iron-Dextran Iron Overload.—

Local effects: Intramuscular injections of iron-dextran were given into the outer portion of the thighs without producing any tenderness or evidence of tissue inflammation. The intraperitoneal injections of Imferon produced no discomfort or evidence of peritoneal irritation.

Generalized effects. No immediate or delayed generalized reactions to injections of iron dextran occurred. Both adult dogs that received intramuscular injections maintained their weight throughout most of the injection period (Fig 1). After approximately 4 months Dog 1 refused to eat, lost weight, and had occasional diarrhea, with benzidine positive feces. These symptoms continued until he died 2½ months later. Anorexia and weight loss were less marked in Dog 2. Nine days before death he developed complete anuria followed by total refusal of food, tarry diarrhea, and *tense abdominal distention.* When death seemed imminent, the animal was killed.

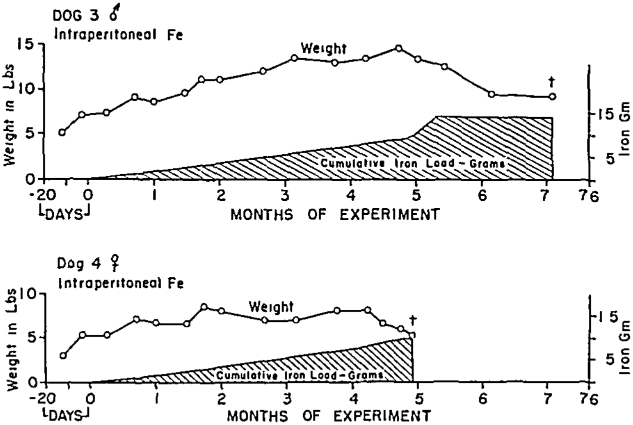


Fig 2.—Correlation of body weight with cumulative iron load in dogs receiving intraperitoneal iron dextran injections.

One puppy (Dog 3) that received intraperitoneal injections of iron dextran doubled his weight during the injection period. However, during the last 2 weeks of injections he lost 2 pounds and continued to lose weight until he died (2 months later). Growth during the injection period was less pronounced in the other puppy (Dog 4) (Fig 2). Her weight fell abruptly during the final period of intensive injections and she died soon afterward. Throughout the experiment both puppies remained vigorous. Mange occurred in each dog; it was mild in Dog 3, but extensive and unresponsive to treatment in Dog 4. There was no premonition of death in either animal.

Total survival time for Dogs 1 and 2 which had received their iron dextran intramuscularly was 227 and 291 days, respectively. Puppies 3 and 4 survived 213 and 148 days from the beginning of their course of intraperitoneal iron dextran injections.

TABLE I. HEMATOLOGIC VALUES OF DOGS RECEIVING IRON-DEXTRAN INJECTIONS

DAY OF EXP'T	R.B.C. (X 10^6)	HGB. (GM./100 ML.)	HCT. (%)	RETIC. (%)	W.B.C. (X 10^3)	DAY OF EXP'T	R.B.C. (X 10^6)	HGB. (GM./100 ML.)	HCT. (%)	RETIC. (%)	W.B.C. (X 10^3)
					A. Intramuscular Iron-Dextran						
		Dog 1							Dog 2		
−20	4.40	7.4	28	0.6	24.2	−20	8.16	18.4	52.5	0.3	20.8
−3	5.29	7.1	23.5	2.2	20.8	−13	8.00	18.7	50.0	2.9	--
9	4.49	9.2	28.5	3.9	--	81	5.96	16.5	43.5	--	--
24	4.97	9.2	32	3.3	--	143	5.33	15.1	40.0	--	15.6
31	4.68	10.3	31	5.4	--	186	4.59	14.5	39.0	--	13.8
38	3.69	11.7	28.5	6.1	--	234	4.46	13.4	38.0	--	21.0
45	3.83	9.6	28	1.2	--	291	4.96	14.5	42.0	--	26.3
53	4.55	12.3	34	18.8	--						
81	4.20	12.5	35	--	--						
133	3.46	9.3	21.5	1.0	16.2						
186	3.72	11.2	32	--	10.8						
					B. Intraperitoneal Iron-Dextran						
		Dog 3							Dog 4		
−16	5.55	9.8	36.5	0.3	19.5	−12	5.24	12.3	33	1.0	26.0
−12	4.87	12.9	35.5	0.4	30.4	−3	4.29	10.2	28	11.9	15.2
−3	5.23	10.5	31.5	2.2	20.3	82	5.47	14.8	41	--	--
82	7.07	16.9	46.5	--	--	132	4.15	10.5	31	--	24.4
186	6.85	17.0	45.0	--	14.9						

Hematologic effects No consistent effect of iron dextran injections on red cell count, hemoglobin value, or packed cell volume was discerned in any of the dogs except Dog 1 This animal, which had an iron deficiency anemia at the beginning of the study, responded to intramuscular iron dextran by a slow rise in hemoglobin and a delayed reticulocytosis No appreciable change in white cell count was observed in any of the dogs (Table I)

Tests of hepatic function Bromsulphalein retention was within normal limits in all dogs both before and approximately 2½ and 4½ months after injections of iron dextran were begun The serum bilirubin was also normal Protein fractionation of serum from Dogs 1 and 2, as tabulated below (Table II), showed a definite fall in total protein, due largely to a fall in the albumin fraction There was a corresponding elevation of serum globulins in the last two samples from Dog 2 Paper electrophoretic analysis confirmed these serum protein changes and demonstrated a diffuse increase of gamma globulins

TABLE II TOTAL AND FRACTIONAL SERUM PROTEIN ANALYSES OF DOGS GIVEN IRON DEXTRAN INTRAMUSCULARLY

ANIMAL AND TIME OF STUDY	TOTAL PROTEIN (GM /100 ML.)	ALBUMIN (GM /100 ML)	GLOBULIN (GM /100 ML)
Dog 1			
Base line	5 9	2 9	3 0
2½ mo	5 9	3 0	2 9
4½ mo	4 5	2 0	2 5
Dog 2			
Base line	6 9	3 9	3 0
2½ mo	5 9	2 8	3 1
4½ mo	4 7	1 9	2 8
7½ mo	5 8	1 5	4 3
9½ mo	5 0	1 5	3 5

Miscellaneous observations Fasting blood sugar values were within normal limits in all dogs A nonprotein nitrogen determination, performed on plasma from Dog 2 just before death, when anuria and tarry diarrhea were present, was 26 mg per 100 ml Serum iron levels are tabulated (Table III) Although they were elevated in every dog, complete saturation of transferrin was not uniformly achieved—a finding which suggests that some of the iron was circulating as iron dextran

Tissue distribution of injected iron (Table IV) The greatest concentration of iron occurred in the liver, spleen, and peripancreatic lymph nodes where values were 50 to 75 times normal Other organs had iron concentrations higher than normal, but the increase was most striking in tissues with a large reticuloendothelial component

Postmortem findings Autopsies were performed within 3 hours of death or after a period of 2 to 6 weeks' storage in a Deepfreeze Since the postmortem findings in all dogs given iron dextran were similar, a single description will be given, with salient exceptions noted

The dogs showed marked emaciation and dull, lusterless hair A scaling, lichenified skin rash was present in Dogs 3 and 4 Dark brown pigmentation of the skin in an area around the sites of injection was present in all animals. There was no free fluid in pleural, pericardial, or peritoneal cavities. The peritoneum and omentum of the dogs that received intraperitoneal injections were stained brown; no adhesions or peritonitis was present. A distinct, rust-brown color was noted in the spleen, lymph nodes, pancreas, and kidneys. The liver was a dark mahogany brown, filled the upper third of the abdomen, and accounted for 6.4 to 13.4 per cent of the animal's weight. Diffuse, black (iron sulfide) pigmentation was present in the intestines of dogs that had undergone postmortem autolysis. The duodenum of Dog 1 showed ascaris infestation. The bladder of Dog 2 was enormously distended and contained several sharp, granular calculi in the bladder neck. The thigh muscles of both dogs that received intramuscular injections were swollen, stained chocolate brown, and the fascial planes were indistinct. No abnormalities of the heart, lungs, great vessels, brain, or other organs were visible.

TABLE III. VALUES OF SERUM IRON, UNSATURATED IRON BINDING CAPACITY, AND TOTAL IRON BINDING CAPACITY IN DOGS GIVEN IRON-DEXTRAN PARENTERALLY

ANIMAL AND TIME OF STUDY	SERUM IRON (μg/100 ML.)	UNSATURATED IRON BINDING CAPACITY (μg/100 ML.)	TOTAL IRON BINDING CAPACITY* (μg/100 ML.)
Dog 1			
Base line	36	341	377
1 mo.	226	64	290
2½ mo.	434	90	524
4½ mo.	693	0	693
Dog 2			
Base line	214	233	447
2½ mo.	1,030	0	1,030
4½ mo.	420	0	420
7½ mo.	334	45	379
9½ mo.	314	4	314
Dog 3			
Base line	119	206	325
2½ mo.	486	9	495
Dog 4			
Base line	173	90	263
2½ mo.	1,720	0	1,720

*Total iron binding capacity is defined as the sum of serum iron and the unsaturated iron binding capacity.

TABLE IV. IRON CONCENTRATION (MG./GM. WET WEIGHT) OF TISSUES OBTAINED AT AUTOPSY OF NORMAL DOGS AND DOGS WITH IRON OVERLOAD

TISSUES	DOG 1	DOG 2	DOG 3	DOG 4	NORMAL DOGS (AVERAGE)
Liver	9.33	12.20	15.08	16.5	0.20
Spleen	5.79	8.23	8.32	4.87	0.24
Pancreas	----	0.13	0.47	0.61	0.06
Kidney	0.39	0.46	0.53	0.41	0.08
Heart	0.13	0.08	0.19	0.38	0.07
Lungs	0.27	0.41	0.18	0.48	0.25
Stomach	0.14	0.05	0.20	----	0.04
Peripancreatic node	12.7	23.8	19.8	18.40	0.06
Bone marrow	2.15	1.31	4.74	----	0.22
Muscle (skeletal)	0.10	0.05	0.163	0.10	0.04
Muscle (skeletal)*	17.15*	15.2*			
Skin (abdomen)	0.38	0.05	0.59	----	0.02
Skin (abdomen)*	----	0.43*	7.56*	1.27*	
Thyroid	----	0.08	----	----	0.06
Adrenal	----	0.42	1.57	----	0.07
Testis	0.18	----	0.50	----	0.01
Eye	0.02	0.01	0.06	----	0.01

*Sample taken from site of iron-dextran injections.

Microscopic examination of the liver showed large clumps of iron filled macrophages (siderophages) aggregated within the sinusoids but relatively few in the fibrous tissue of the portal areas Only occasional small granules of iron were seen in either the hepatic parenchymal cells or the bile duct epithelium Slight atrophy of liver cell cords in the regions of maximal iron deposition was noted, there was no fibrosis or cirrhosis (Fig 3)

The spleen of each dog contained large numbers of iron granules distributed almost exclusively within macrophages of the sinusoids in the red pulp, but few or none were found in the follicles and the capsule Large deposits of granular iron in the fibroadipose tissue in the hilum of the spleen were seen in 2 animals (Fig 4)

Peripancreatic lymph nodes of all dogs and the inguinal and iliac nodes of dogs receiving iron dextran intramuscularly showed heavy infiltration of iron in the pulp but little in the follicles Tremendous deposits of iron occurred in the sinusoidal areas and the perinodal fibroadipose tissue Paratracheal and axillary nodes were pigmented but to a smaller extent No fibrosis of any lymph node was seen

No abnormalities were noted in the pancreas Acinar and islet tissue appeared normal and there was no fibrosis Only an occasional siderophage was present in the interstitial tissues

The kidneys showed moderately heavy accumulations of siderophages in the fibroadipose tissue about the pelvis but relatively little iron within the kidney substance There was a moderate amount of finely granular iron in the glomerular tufts and in the epithelium of segments of the convoluted tubules (Fig 5) The exact location of this segmental tubular involvement was not clear, but it appeared to be principally in the proximal segments No histologic abnormalities other than the iron pigment were noted The urinary bladder of Dog 3 showed numerous iron granules within macrophages in the adipose tissue beneath the serosa Smaller amounts of iron were scattered in the interstitial tissue throughout the muscularis, particularly around blood vessels In Dog 2, which had obstruction of the bladder neck due to calculi, there were focal areas of erosion in the bladder epithelium with underlying inflammation and hemorrhage into the lamina propria

No abnormalities and no iron deposition were observed in the heart of any dog except Dog 3 This animal showed a moderate number of scattered siderophages in the connective tissue beneath the epicardium and endocardium and between the muscle fibers Iron in the lungs was confined primarily to macrophages in alveolar septa which appeared to have been trapped in alveolar capillaries A few siderophages were present in the fibrous tissue around the bronchi, others lay free in the alveolar lumens Bronchitis and edema were present in Dog 4, and Dog 1 had an extensive bronchopneumonia

Sections of bone marrow taken from ribs, sternum, vertebral body, and femur all showed moderate to heavy concentrations of granular iron in clumps, but no detectable cellular abnormality and no fibrosis The structure of bone and cartilage was normal

No iron was seen within the brain substance or in the choroid plexus Rare macrophages in the leptomeninges contained small granules of iron The eyes of Dog 2 showed no abnormalities

Adrenals, thyroid, pituitary, and salivary glands showed no structural abnormalities Small granules of iron were present in isolated macrophages of the interstitial connective tissue, but *none was seen within epithelial cells*

Sections of muscle at sites of iron dextran injection showed extensive areas of dense infiltration with macrophages containing large iron granules These masses of iron displaced the muscle fibers but seemed to have incited no fibrous or foreign body reaction The muscle fibers themselves appeared to be normal except for atrophy in some areas Muscles in areas distant from sites of iron injection were normal and showed no iron deposition Sections of diaphragm from dogs receiving iron dextran intraperitoneally showed small to moderate numbers of coarse iron granules in the interstitial tissue between muscle bundles

Siderophages in moderate numbers were scattered throughout the upper corium and were particularly concentrated around blood vessels and skin appendages Considerable

Fig. 3

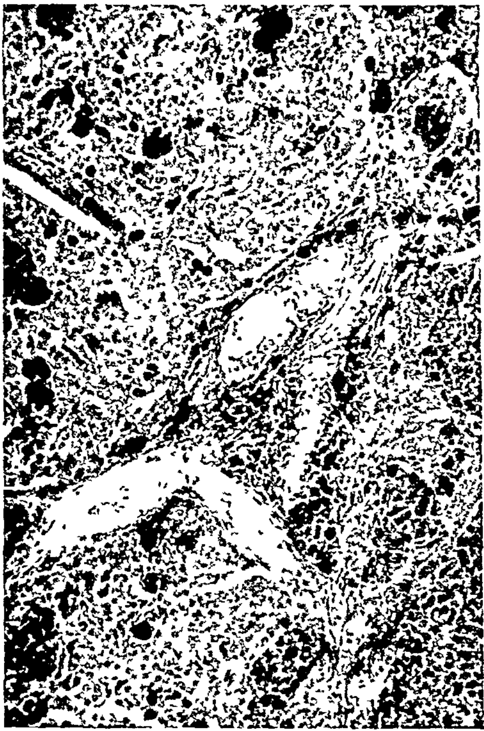
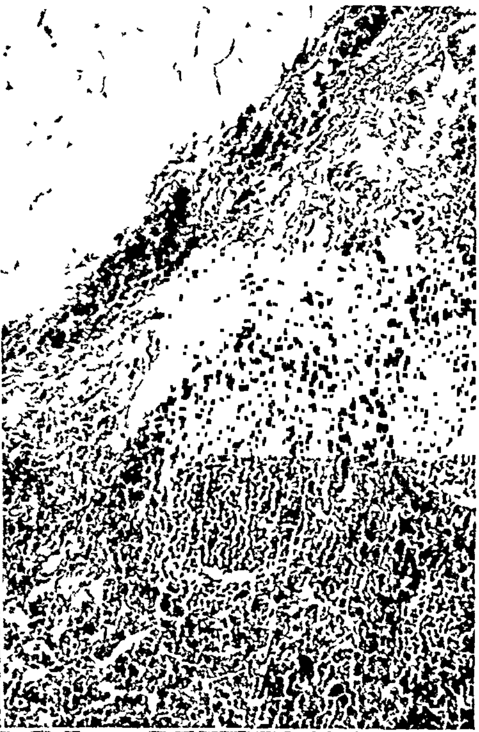


Fig. 4.



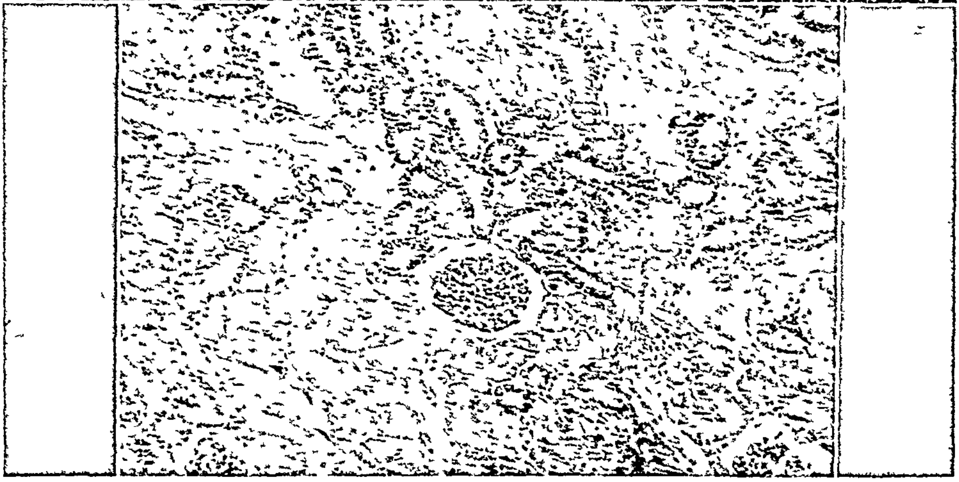


Fig. 5

Fig. 3.—Liver from Dog 2 which received iron-dextran intramuscularly. The iron, which shows as black masses in the illustration, is concentrated in clumps of macrophages in the sinusoids and portal space. There is no fibrosis, injury to hepatic cells, or iron in bile duct epithelium, and only a small amount of iron in hepatic cells. Hematoxylin and eosin stain. (Magnification ×140; reduced 1/6.)

Fig. 4.—Spleen from Dog 2. The dark masses of iron are present in macrophages principally in the red pulp about the follicles but also in areolar tissue adherent to the capsule. Hematoxylin and eosin stain. (Magnification ×140; reduced 1/6.)

Fig. 5.—Kidney from Dog 2. Granules of iron are present in renal tubular cells and in endothelial cells of the glomerular tufts without inducing appreciable alterations in these tissues. Gomori's iron stain. (Magnification ×140; reduced 1/6.)

keratinization of the epithelium and plugging of the hair follicles with coarse clumps of keratin were seen.

Scattered siderophages were seen in the interstitial tissue of the testes in all dogs. The testes of Dog 1 showed some arrest of spermatogenesis, as evidenced by absence of spermatids, but there was no atrophy or fibrosis. Dog 2 showed active spermatogenesis. Testicular fibrosis and atrophy were present in the half grown dog (No 3). This animal also had moderate concentrations of siderophages throughout the fibrous tissue of the tunica *vaginalis and epididymis. Except for scattered siderophages throughout the stroma of the* prostate, this organ was normal in all animals.

There were slight to moderate numbers of siderophages in the connective tissue beneath the epithelium of the tongue in Dogs 2 and 3. Siderophages in the fibrous tissue of the lamina propria and adventitia of the esophagus were present only in the dogs that had received iron dextran intraperitoneally. In the stomach and intestines the connective tissue of the lamina propria, muscularis, and subserosa showed moderate numbers of macrophages containing iron. Dogs 3 and 4, which had received intraperitoneal injections, showed larger *concentrations of iron in the serosa than the animals that had received intramuscular injections.* No epithelial iron deposition or lipochrome pigmentation was seen.

Huge deposits of hemosiderin accompanied by granular eosinophilic debris that may have represented the residue of edema or foreign body reaction were present in remnants of fibroadipose tissue of the omentum in the dogs injected intraperitoneally with iron dextran. The gall bladders of these dogs showed large clumps of siderophages in the subserosal connective tissue and lymphatic spaces.

Certain findings warrant emphasis. There was no significant difference in iron distribution correlated with the route of iron dextran administration. Iron deposition was confined almost exclusively to cells of the reticuloendothelial system. Parenchymal iron deposits were small and confined to epithelial cells of the liver, bile ducts, and renal tubules. Even when present in tremendous concentration, the iron produced no fibrous tissue or foreign body reaction there was no cirrhosis of the liver or pancreatic fibrosis. Testicular atrophy was present in one dog and decreased spermatogenesis was observed in another, however, the amount of iron in the testes of these dogs was small, and the testicular changes were unlike those described by Nissim in rats injected with iron dextran. The histologic appearance of the tissues failed to show the cause of death. The bronchitis and bronchopneumonia in 2 dogs were probably terminal phenomena.

B Saccharated Iron Oxide Iron Overload.—Dog 5, injected intravenously with saccharated iron oxide, had been a member of the colony for 2 years and had been used in several earlier experiments. One of these was an assay of the effects of 4 aminofolic acid with and without concomitant folic acid, no ill effects were seen. Prolonged phenylhydrazine induced hemolytic anemia was subsequently produced in this dog, but he was allowed to recover completely before beginning this study. One month before iron injections were begun a laparotomy was performed, and biopsies for chemical iron analysis and histologic appraisal were taken of liver, spleen, pancreas, kidney, mesenteric lymph node, skeletal muscle, and skin. The histologic appearance of these tissues was normal except for the following (1) iron was present in increased *amounts principally* in Kupffer cells of the sinusoids of the liver and in a few macrophages of the portal areas, (2) numerous siderophages were present in the red pulp of the

Fig. 3.

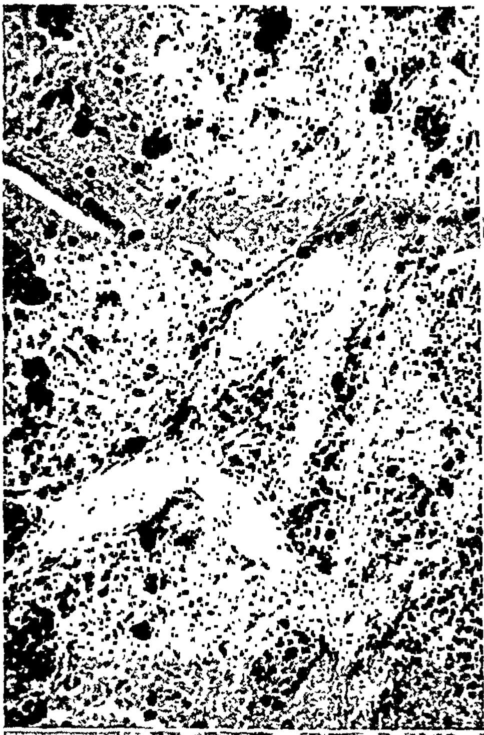
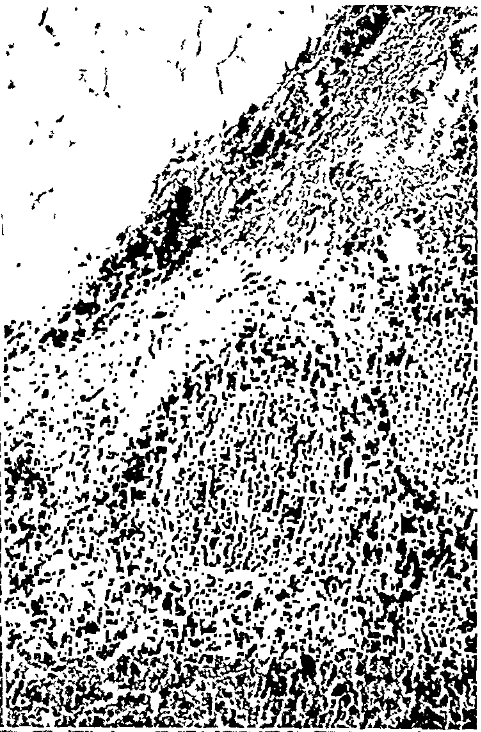


Fig. 4.



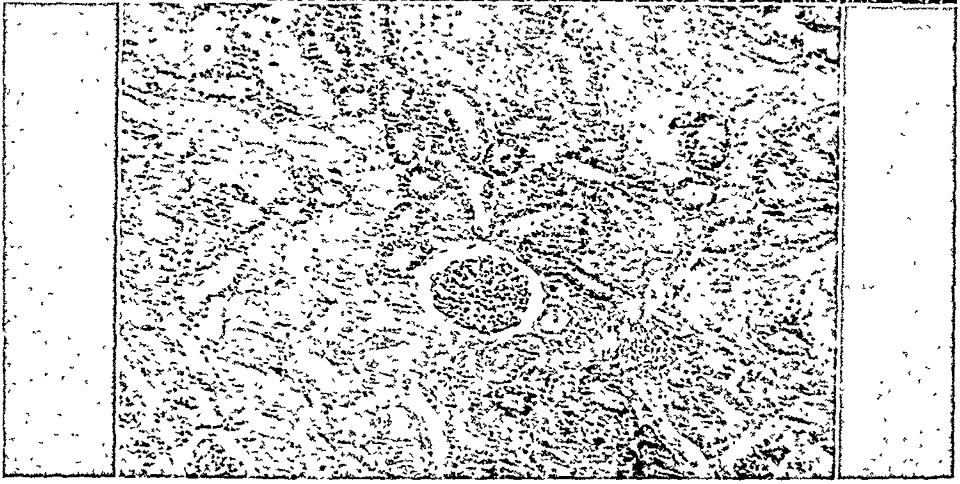


Fig. 5.

Fig. 3.—Liver from Dog 2 which received iron-dextran intramuscularly. The iron, which shows as black masses in the illustration, is concentrated in clumps of macrophages in the sinusoids and portal space. There is no fibrosis, injury to hepatic cells, or iron in bile duct epithelium, and only a small amount of iron in hepatic cells. Hematoxylin and eosin stain. (Magnification ×140, reduced ⅙.)

Fig. 4.—Spleen from Dog 2. The dark masses of iron are present in macrophages principally in the red pulp about the follicles but also in areolar tissue adherent to the capsule. Hematoxylin and eosin stain. (Magnification ×140; reduced ⅙.)

Fig. 5.—Kidney from Dog 2. Granules of iron are present in renal tubular cells and in endothelial cells of the glomerular tufts without inducing appreciable alterations in these tissues. Gomori's iron stain. (Magnification ×140; reduced ⅙.)

weight loss, emaciation, and unkempt, lusterless hair were prominent at 3 months. Supplementation of his regular diet with horse meat resulted in a temporary weight gain and improved appearance. At 5 months, however, ulcers appeared in the skin of the face and forelegs and failed to heal despite the use of penicillin injections, Aureomycin by mouth, and local antibiotic salves. Two weeks later ascites appeared, then edema of the feet and legs. Attempts to alleviate the ascites and edema by means of repeated injections of mercurial diuretics and 3 transfusions of plasma from a normal dog were unsuccessful. The animal died 13 days after his last iron injection, 7 months after the start of the experiment.

Laboratory observations. Since the effects of saccharated iron oxide on blood counts, serum iron, hepatic function, and blood sugar levels in an earlier series of dogs have been reported,[1] only a minimum of observations was made on this dog (Table VI). Progressive anemia began less than 2 months after the start of injections and continued until death. During the last 2 weeks of life the serum albumin, the total protein, and the free and esterified cholesterol were low, suggesting functional impairment of the liver. The serum iron did not rise to levels as high as those of dogs in another series which received Ferrivenin.[1] No evidence of diabetes was observed.

TABLE VI. LABORATORY DATA OF DOG 5 GIVEN SACCHARATED IRON OXIDE INTRAVENOUSLY

DAYS OF I.V. IRON INJECTIONS	RED CELL COUNT (MILLIONS)	HEMOGLOBIN (GM./100 ML.)	CELL VOLUME PER CENT	SERUM IRON (μg/100 ML.)
0	--	16.6	47	---
59	5.53	13.2	36	-
86	4.08	11.8	34	810
100	3.65	10.5	30	
124		12.4	35	690
136	4.07	12.3	38	-
177	3.86	10.7	32	543
198	3.40	8.6	26	550
205	2.70	8.0	24	690
208	2.70	7.4	21	789

Miscellaneous tests were as follows: Day 198: total serum proteins 4.9 Gm./100 ml.; albumin 1.8 Gm./100 ml.; serum cholesterol (total) 47 mg./100 ml.; free 22 mg./100 ml. Day 208: total serum proteins 4.9 Gm./100 ml.; albumin 2.0 Gm./100 ml.

Distribution of injected iron. The total iron found in the tissues of this dog was estimated at 28.8 of the 29 Gm. injected (Table V). An additional 3.2 Gm. of iron probably was present in the bone marrow, lungs, and other tissues not assayed, bringing the total recovery to 32 Gm. or 3 Gm. more than was injected. The most plausible explanation for this discrepancy is that this animal had increased body iron stores before the beginning of this study (as a result of receiving a prolonged course of phenylhydrazine to induce anemia in a previous study). The distribution of iron was similar to that of the dogs receiving iron dextran and showed huge accumulations in liver, spleen, and peripancreatic lymph nodes and very little iron in tissues having a small reticuloendothelial component.

Postmortem findings. Gross examination revealed generalized muscle wasting, distention of the abdomen, and edematous extremities. Infected ulcers were present on the face and hind legs; the hair was dry and lusterless; the skin and mucous membranes were rust-colored. Pink ascitic fluid (1,450 ml.) was present, but there was no free fluid in the pleural or pericardial cavities. The liver was large, firm, and chocolate brown. The spleen and peripancreatic lymph nodes were dark brown; the pancreas and kidneys were a lighter tan color. Except for a rusty pigmentation of the mucosa of the esophagus and stomach and the presence of a tapeworm in the upper ileum, the gastrointestinal tract was normal. Examination of the heart showed brown plaques on the endocardial surfaces of the mitral, aortic, and pulmonic valves and similar plaques in the aorta extending to a point 1 cm. below the origin of the subclavian artery. No abnormalities were seen on gross examination of the other organs.

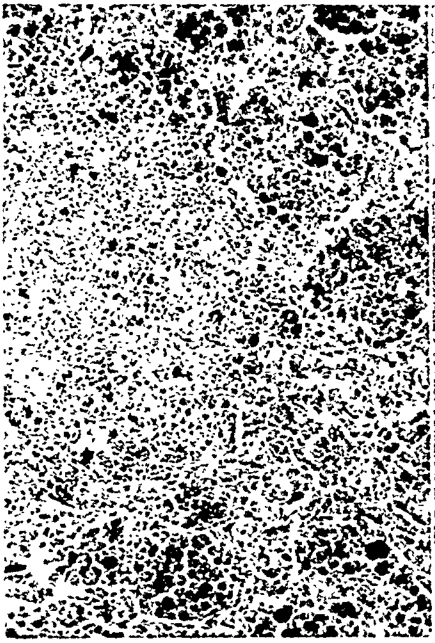


Fig. 7.

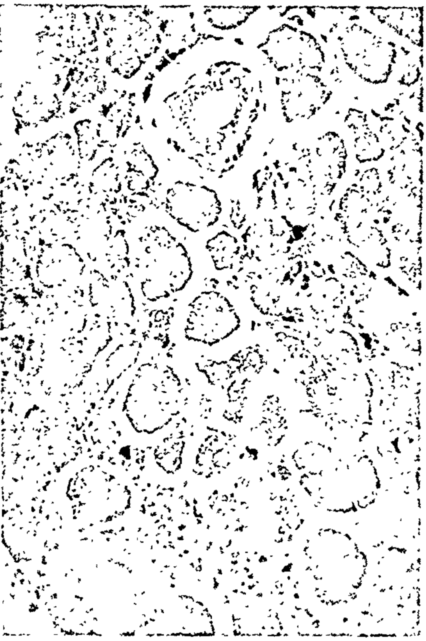


Fig. 8.

Fig. 7.—Liver from Dog 5 which received saccharated iron oxide intravenously. Dark masses of iron are distributed in a manner similar to those in Fig. 3. There is a large area of *bland necrosis* with *iron granules in its debris*. *Hematoxylin and eosin stain.* (*Magnification* ×140; reduced ¼.)

Fig. 8.—Pancreas from Dog 5. There is no fibrosis and the epithelium of neither the islets nor the acini shows any changes. Very little iron is present, mostly as dark masses in the interstitial tissue. Gomori's iron stain. (Magnification ×230; reduced ¼.)

Microscopic examination showed the same general pattern of iron distribution as has been described previously.[1] The amount of iron deposition was greater, reflecting the three- to sixfold increase in dosage, but the predominant *reticuloendothelial distribution* and absence of fibrous tissue reaction to the iron were identical. Because of the similarity to the other dogs, only a description emphasizing differences will be given.

Iron deposits in the liver were confined to huge clumps of macrophages within the sinusoids and to smaller groups of distinct granules in the cytoplasm of the parenchymal cells; the bile duct epithelium was practically free of iron. Of particular interest were areas of focal necrosis 50 to 250 μ in diameter scattered throughout the liver (Fig. 7). In these areas there were destruction of liver cells, a slight increase in wandering macrophages, and a few polymorphonuclear leukocytes. The reticulum appeared to be preserved, and iron was present in macrophages or as free granules where cells had been destroyed. The remainder of the liver showed no increase in fibrous tissue or other abnormalities.

The spleen was massively infiltrated with siderophages predominantly within the reticulum of the red pulp, with little iron in the follicles. As was true of most organs examined, occasional iron containing macrophages were present beneath the endothelium of blood vessels. The architecture of the pancreas was normal and showed no fibrosis. Iron deposition in this organ spared epithelial cells and was confined to interstitial tissue (Fig 8).

The kidney glomeruli showed a peculiar and irregular thickening of the capillary basement membranes within the tufts, and contained relatively large accumulations of iron granules. This thickening seemed to have decreased the general vascularity of the glomerular tufts, although many capillaries were wide open and filled with erythrocytes. Small amounts of finely granular hemosiderin were present in the tubular epithelium, particularly of the proximal tubules (Fig 9).

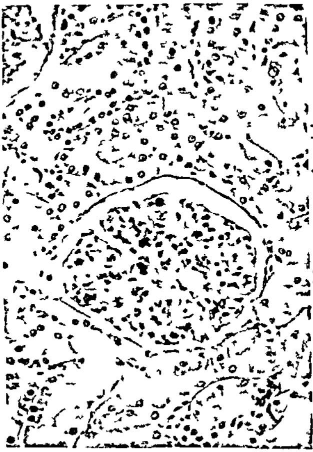


Fig 9

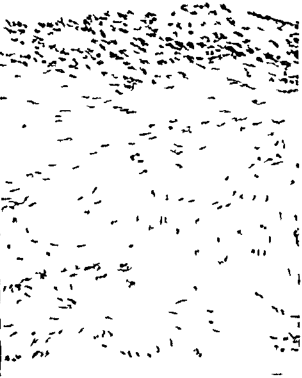


Fig 10

Fig 9—Kidney from Dog 5. The capillary basement membranes of the glomeruli are irregularly thickened and cause constriction of the lumens of the capillaries. Dark masses of iron are also present in the glomeruli but only fine granules are present in the epithelium of a few tubules. Hematoxylin and eosin stain. (Magnification ×70, reduced ¼.)

Fig 10—Aorta of Dog 5. An intimal plaque from just above the aortic valve shows the accumulation of macrophages filled with iron granules throughout its substance. Hematoxylin and eosin stain. (Magnification ×140, reduced ¼.)

In the heart siderophages were present almost exclusively in interstitial tissues of the myocardium and epicardium. In addition, there was deposition of siderophages 4 or 5 cells thick, in the loose fibrous tissue immediately beneath the endocardium and the intima of the aorta which accounted for the plaques seen on gross examination (Fig 10). Iron in the lungs was present only in siderophages trapped in alveolar capillaries.

Iron in the gastrointestinal tract was present mainly in macrophages in the lamina propria, with small collections of siderophages in the submucosa of the stomach and the subserosa of the small intestines. There was a brownish yellow, refractile pigment that did not stain for iron but did stain positively with basic fuchsin in the interstitial tissue of the muscularis. This lipochrome or "hemofuscin pigment" was present in greatest quantities in the lower ileum but was also seen in the stomach, jejunum, colon, and interstitial tissue of the pancreas.

The testes showed scattered iron-containing macrophages in the interstitial tissue between the tubules but no atrophy of the tubules. Rare siderophages were present in the interstitial tissues of pituitary, adrenal and salivary glands; no pigmentation of the epithelium was seen.

Sections of vertebral and femoral bone marrow showed erythroid hypoplasia with slight fibrosis and a heavy infiltration of siderophages. Lymph nodes were filled with huge aggregates of siderophages, some of which spilled into surrounding alveolar tissue and a few into the interstitial areas of the compressed follicles.

Relatively small amounts of iron were found in muscle and skin, in siderophages about the blood vessels and hair follicles. A section of a chronic ulcer of the foreleg showed no deposition of iron in association with the inflammatory infiltrate.

No iron was seen in brain substance. A small number of siderophages was present in cells of the choroid plexus and the leptomeninges. The eyes contained moderate amounts of hemosiderin in the ciliary epithelium, in the stroma of the ciliary body, and in the choroid. The pigment epithelium showed swelling and degeneration, but the retina was free of abnormalities.

As in the dogs that received iron-dextran, this dog showed iron deposits primarily in the cells of the reticuloendothelial system, with parenchymal deposition only in the liver and kidney tubules. No cirrhosis or fibrosis was produced by the iron. Four findings in Dog 5 did not appear in the dogs which received iron-dextran. First, there were small scattered areas of focal necrosis of the liver of unknown significance. Second, lesions of the glomeruli similar to those described in other species of experimental animals as being due to small emboli caused by intravascular precipitation of saccharated iron oxide were observed.[3, 4] (The extensive renal lesions described by Ellis[5] in rats injected with saccharated iron oxide were not seen.) Third, hemofuscin-like pigment was found in the pancreas and the muscularis of the gastrointestinal tract. The same pigment has been described in other dogs injected with saccharated iron oxide.[1] Golberg and associates[6] have suggested that a similar pigment produced in iron-dextran injected rats is related to a localized vitamin E deficiency. Finally, the iron deposits in various segments of the eye seemed to mark the early stages of degenerative ocular changes which have been described in other dogs injected with large amounts of iron.[7]

DISCUSSION

The cumulative dose of iron needed to produce recognizable toxic effects in these dogs was approximately 2 Gm. per kilogram of body weight; the lethal dose was 2.5 to 3.3 Gm. per kilogram. The dose was independent of the route of parenteral administration or type of iron compound used. The only report of iron overload of similar magnitude in dogs was that of Finch and Finch[8] who gave saccharated iron oxide and observed hepatic toxicity at doses of 2 Gm. per kilogram in dogs that survived for 2 years.

The way that the excess tissue iron produced the observed harmful effects is obscure. The anorexia, apathy, weight loss, and fall in serum albumin concentration do not implicate any specific mode of iron toxicity. They could be ascribed to malnutrition, but this label gives no insight into the role played by the iron deposits. The course of the dogs that received iron dextran suggests a saturation phenomenon. Early in the course of iron injections no ill effects were observed. After about 4 months, at levels of iron greater than 2 Gm. per kilogram, there seemed to be a breakdown of tolerance to the massive iron deposits with the appearance of toxic manifestations.

Finch and Finch have emphasized that iron introduced parenterally is largely captured by cells of the reticuloendothelial system so that the parenchymal cells are protected from damage and fibrous tissue response. If this explanation were wholly correct, one might expect that the terminal toxic effect could be correlated with a spillover of iron into parenchymal cells after the capacity of the reticuloendothelial system had been exceeded. No morphologic evidence for this phenomenon was found.

In addition to the other changes ascribed to malnutrition, the dog that received saccharated iron oxide developed ascites and anasarca, thereby suggesting liver failure as the cause of death. There is no doubt that the liver bears the brunt of iron deposition and may, therefore, sustain the greatest injury. Extremely low free and esterified serum cholesterol and low serum albumin concentrations in this animal were the only measured evidence of hepatic dysfunction, other functions such as bile pigment metabolism seemed to be reasonably intact. Examination of the liver at death gave little morphologic evidence of injury. Except for scattered, small areas of focal necrosis and the massive reticuloendothelial iron deposits, the liver did not appear seriously damaged. In the other dogs, even fewer abnormalities of the liver were noted.

Since there was no acceptable morphologic explanation in the liver or other tissues for the deterioration and death of these dogs one may speculate about possible biochemical alterations that may have been involved. Little is known about iron toxicity at the molecular level. Ferritin, which is a usual accompaniment of hemosiderin in tissue iron storage can under certain circumstances produce severe vasodepression and may lead to death.[9] Such a death is catastrophic, unlike the slow deterioration observed in these dogs. As another possibility, iron, a heavy metal, shares the property of binding sulfhydryl groups and may thus interfere with certain enzyme systems. Glycolysis of tissue homogenates is inhibited by ferrous sulfate,[10] and it has been postulated that pyruvate formation[11] and "enzymes in the Kreb's cycle"[1] are inhibited by iron in toxic amounts. The methods used in this experiment were not designed to test these hypotheses. It seems likely, however that systemic toxic effects of iron overload invariably depend on interference with enzyme systems, whether or not morphologic changes are produced.

These data obtained from dogs may apply to human beings because of the many similarities of iron metabolism of the two species. The results emphasize the fact that excessive tissue iron deposits can lead to death by processes independent of the fibrosis, cirrhosis, and diabetes seen in human subjects with idiopathic hemochromatosis. Although no instance has yet been reported of harmful iron overload in human subjects as a result of administration of either saccharated iron oxide or iron dextran, this possibility cannot be ignored. Even the remote potentiality of fatal iron overload, as proved possible by these experiments, cautions against the indiscriminate use of these easily administered iron preparations.

SUMMARY AND CONCLUSIONS

The cumulative dose of parenterally administered iron required to produce death within 5 to 10 months in each of 5 dogs ranged from 2.5 to 3.3 Gm. per kilogram of body weight; toxic effects began to appear after 2 Gm. per kilogram had been given. The size of the lethal dose was not affected by the type of iron compound used (iron-dextran or saccharated iron oxide) or by the route of administration (intramuscular, intraperitoneal, or intravenous).

Massive tissue iron deposition occurred almost exclusively in cells of the reticuloendothelial system, with little parenchymal involvement. There was no fibrous tissue response to iron deposits and no evidence of hemochromatosis.

The mechanism by which the toxic effects were produced and the cause of *death of these dogs have not been defined.*

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SOME PHYSICOCHEMICAL PROPERTIES OF THE VITAMIN B_{12} BINDING SUBSTANCES OF NORMAL AND CHRONIC MYELOGENOUS LEUKEMIC SERA

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THE serum in chronic myelogenous leukemia (CML) has an increased concentration of vitamin B_{12}[1, 2] and an increased capacity to bind vitamin B_{12} as measured in vivo[3, 4] or in vitro[1, 2, 5]. By microbiologic assay of the electrophoretically separated fractions of normal and CML serum, vitamin B_{12} was found bound to the alpha 1 and alpha 2 globulins.[1, 6] The same proteins were also involved in the in vitro binding of added vitamin B_{12}.[1, 6] When cobalt^{60} labeled vitamin B_{12} ($B_{12}CO^{60}$) sufficient to saturate normal serum is added in vitro it is bound chiefly to the beta and alpha 2 globulins as measured by dialysis techniques. On the other hand, the high levels of added $B_{12}CO^{60}$ bound by CML serum is found on the alpha 1 and alpha 2 globulins.[8] In the present study we have investigated (a) the nature of the substances in normal and CML sera that bind $B_{12}CO^{60}$ and (b) some physicochemical characteristics of this binding reaction.

A preliminary report of this study has been published.[9]

METHODS

Serum.—Venous blood was collected from fasting control subjects and from CML patients. The blood was allowed to clot at room temperature and, 2 to 3 hours later, the serum separated and stored at 20° C until used. Control sera were routinely pooled in groups of 3 to 4 and CML sera were occasionally pooled before use. Control subjects were laboratory personnel and patients in the hospital suffering from various types of functional disorders. CML sera were from patients who were in clinical and hematologic relapse.

Unsaturated Vitamin B_{12} Binding Capacity of Serum (UBBC).—The ability of normal or CML serum to bind added $B_{12}CO^{60}$ (UBBC) was determined by a slightly modified equilibrium dialysis technique described previously.[8] One milliliter of saline containing 29 mμg of $B_{12}CO^{60}$ was added to 4 ml. aliquots of normal and 1 or 2 ml. aliquots of CML sera. This amount of $B_{12}CO^{60}$ was in excess of the binding capacity of all sera except for one CML serum in which 58 mμg per milliliter of serum was required. The mixture was allowed to interact in a Visking dialysis bag for one hour at room temperature. Variations in the amount of serum used and in the total volume of the mixture did not affect the results provided an excess of $B_{12}CO^{60}$ was present. The serum $B_{12}CO^{60}$ mixture was then dialyzed for 48 hours at 4 to 6° C against 300 ml. of 0.062 M phosphate buffer, pH 7.3. A protein free blank containing buffer instead of serum was run concomitantly. The method of sample counting and the calculation of the UBBC from the experimental results was similar to that described previously.[8] All studies were performed in triplicate except for some experiments using CML sera

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where duplicate samples only were analyzed. If duplicate or triplicate samples disagreed by more than 15 per cent the entire experiment was repeated. When serum whose binding capacity was saturated with added $B_{12}CO^{60}$ was required, the serum $B_{12}CO^{60}$ mixture was dialyzed against large volumes of 0.062 M phosphate buffer, pH 7.3 for 96 hours. In the estimation of the UBBC from such samples no correction for a protein-free blank was necessary.

Microbiologic Assay Measurements.—The *Euglena gracilis* assay was employed in these studies.[10] In this assay, one volume of diluted serum was added to an equal volume of basal growth medium. The pH of the basal medium as well as the serum-basal medium mixture is 3.6. The composition of this medium in grams per liter of water is as follows: H_3BO_3, 0.071; KH_2PO_4, 0.100; $MgSO_4 \cdot 7H_2O$, 0.100; L-glutamic acid, 10.000; DL-malic acid, 2.000; $CaCO_3$, 0.122; $ZnSO_4 \cdot 7H_2O$, 0.888; $MnSO_4 \cdot H_2O$, 0.031; $FeSO_4 \cdot 7H_2O$, 0.005; $CuSO_4 \cdot 5H_2O$, 0.004; $Na_2MoO_4 \cdot 2H_2O$, 0.005; $CoSO_4 \cdot 7H_2O$, 0.019; thiamine, 0.002. In this assay "free vitamin B_{12}" is defined as the vitamin available to *Euglena gracilis* without preliminary heating of the serum-basal medium mixture and "total vitamin B_{12}" as the vitamin available after heating of the serum-basal medium mixture at 100° C. for 17 minutes.[10] The "free" and "total" vitamin B_{12} activity was measured in: (A) serum to which excess $B_{12}CO^{60}$ had been added and had then been exhaustively dialyzed against 0.062 M phosphate buffer at pH 7.3. This serum contained the endogenous vitamin B_{12} plus the additional exogenous $B_{12}CO^{60}$ which was nondialyzable at pH 7.3. (B) Aliquots of the same serum to which $B_{12}CO^{60}$ had not been added but which had been dialyzed as serum A. This serum contained only the endogenous vitamin B_{12} activity. The amount of nondialyzable exogenous $B_{12}CO^{60}$ that was measured as "free" or "total" B_{12} in the *Euglena gracilis* assay could then be calculated from the following:

1. "Free" B_{12} activity of the exogenous $B_{12}CO^{60}$ = "Free" B_{12} activity (A) − "free" B_{12} activity (B)
2. "Total" B_{12} activity of the exogenous $B_{12}CO^{60}$ = "Total" B_{12} activity (A) − "total" B_{12} activity (B)

pH Experiments.—Sera were dialyzed for 72 hours against frequent changes of 0.15 M saline at 4° C. Aliquots of this sera were placed into dialysis bags and allowed to dialyze against buffers of varying pH values at constant ionic strength (0.15). In these experiments the $B_{12}CO^{60}$ was added to the dialysate rather than inside the bag. After dialysis for 72 hours the UBBC was determined. After dialysis, sera dialyzed against the pH 5.5 and 4.5 buffers became turbid but gross precipitation did not occur. The following buffers were used: (1) 0.15 M sodium borate, pH 9.2; (2) 0.062 M sodium phosphate, pH 7.3; (3) 0.15 M sodium acetate pH 5.5; (4) 0.15 M sodium acetate, pH 4.5; and (5) 0.15 M sodium formate, pH 3.6.

Serum containing bound $B_{12}CO^{60}$ was prepared by exhaustive dialysis as described above, and the UBBC was then determined. Aliquots of such sera were mixed with two volumes of the *Euglena gracilis* basal medium. The serum-basal medium mixture was then dialyzed against large volumes of the basal medium (pH 3.6). As a control two volumes of 0.062 M phosphate buffer pH 7.3 were added to aliquots of the same serum and the contents dialyzed against this buffer. The UBBC of the acidified and control serum were then redetermined after dialysis. The UBBC of the acidified serum listed in the Results was corrected for the slight decrease in the UBBC of the control serum.

Seromucoid Preparation.—The seromucoid, i.e., the fraction of serum soluble in 0.2 M sulfosalicylic acid[11] was prepared. One volume of 0.4 M sulfosalicylic acid was added to one volume of serum. One minute after precipitation of the serum proteins, the contents were centrifuged at 1,000 g 4° C., for 45 minutes. The supernatant containing the seromucoid was separated and then neutralized with 1 N NaOH. The seramucoid was precipitated using one-half volume of 5 per cent phosphotungstic acid in 2 N HCl.[11] $B_{12}CO^{60}$ binding by the neutralized supernatant both after sulfosalicylic acid precipitation and after phosphotungstic acid precipitation is shown in Table I. $B_{12}CO^{60}$ binding occurred in the sulfosalicylic acid supernatant containing the seromucoid, but was virtually nil in the phosphotungstate supernatant in which the seromucoid was absent. Thereafter, $B_{12}CO^{60}$ binding by the neutralized sulfosalicylic acid supernatant was measured and taken to represent $B_{12}CO^{60}$ binding of the seromucoid.

TABLE I. THE BINDING OF $B_{12}CO^{60}$ BY THE SEROMUCOID FRACTION OF CML SERUM

	UBBC (mμg/ML.)
(A) CML serum	14.92
(B) Seromucoid fraction*	9.46
(C) Seromucoid supernatant†	0.01

*The supernatant after precipitation of the serum proteins from (A) with 0.2 M sulfosalicylic acid.

†The supernatant after precipitation of the seromucoid fraction from (B) with 5 per cent phosphotungstic acid in [illegible] N HCl.

The Effects of Heat.—Whole serum and the seromucoid fraction were heated for 3 minutes at 69 to 73° C. CML seromucoid was also heated for 10 minutes at 100° C. in a boiling water bath. All samples were then cooled to room temperature and $B_{12}CO^{60}$ binding measured.

RESULTS

Exchangeability of Bound $B_{12}CO^{60}$.—Carrier vitamin B_{12} was allowed to interact for one hour with serum containing bound $B_{12}CO^{60}$, and the UBBC was then redetermined by dialysis (Table II). No appreciable exchange of bound $B_{12}CO^{60}$ with carrier B_{12} was noted in either normal or CML sera. The rapidity of binding is indicated by the fact that carrier B_{12} failed to dislodge $B_{12}CO^{60}$ when the carrier vitamin was added to the serum one minute after the addition of the radiovitamin (Table III).

TABLE II. THE FAILURE OF CARRIER VITAMIN B_{12} TO EXCHANGE WITH $B_{12}CO^{60}$ PREVIOUSLY BOUND TO SERUM

SERUM	UBBC (mμg/ML.)	
	INITIAL BINDING	CARRIER B_{12}*
Normal	1.40	1.33
CML	12.46	12.79

*Four micrograms of carrier vitamin B_{12} was allowed to interact with sera containing previously bound $B_{12}CO^{60}$ for 1 hour at room temperature following which the UBBC was redetermined by dialysis.

TABLE III. THE RAPIDITY OF $B_{12}CO^{60}$ BINDING BY NORMAL AND CML SERUM

SERUM	UBBC (mμg/ML.)	
	CONTROL	CARRIER B_{12}*
Normal	1.53	1.43
CML	18.10	17.20

*One minute after the addition of 29 mμg $B_{12}CO^{60}$ to the serum 500 mμg of carrier vitamin B_{12} was added. One hour later dialysis of all samples was begun.

Comparison of Euglena gracilis and Dialysis Binding Methods.—The radiovitamin bound to serum by dialysis at pH 7.3 was measured by the *Euglena gracilis* assay method. In normal sera 60 to 77 per cent of the radiovitamin nondialyzable at pH 7.3 was measured as "free" or unbound by *Euglena gracilis* assay whereas only 9 to 3 per cent was so measured in CML sera (Table IV).

However, expressed in absolute terms (millimicrograms per milliliter of serum), the amount of "free" $B_{12}CO^{60}$ was similar for both types of sera (Table IV). Since the serum is first acidified in the performance of the *Euglena gracilis* assay, the effect of an acid milieu on the $B_{12}CO^{60}$ nondialyzable at pH 7.3 was investigated.

TABLE IV. THE ABILITY OF EUGLENA GRACILIS TO UTILIZE NONDIALYZABLE $B_{12}CO^{60}$ OF NORMAL AND CML SERA

SERUM	"TOTAL" $B_{12}CO^{60}$ CONCENTRATION (mμg/ML.)	"FREE" $B_{12}CO^{60}$ CONCENTRATION (mμg/ML.)	$\frac{\text{"FREE" } B_{12}CO^{60}}{\text{"TOTAL" } B_{12}CO^{60}} \times 100$ (%)
Normal			
1	2.11	1.62	77
2	2.88	1.72	60
3	3.30	2.60	77
CML			
1	19.30	2.50	13
2	16.00	1.42	9
3	12.23	1.53	13

"Free" and "Total" $B_{12}CO^{60}$ in terms of *Euglena gracilis* assay as described in Methods.

TABLE V. THE EFFECT OF ACIDIFICATION ON THE NONDIALYZABLE $B_{12}CO^{60}$

SERUM	UBBC AT pH 7.3 (mμg/ML.)	UBBC AT pH 3.6* (mμg/ML.)	DECREASE IN UBBC AT pH 3.6	
			mμg/ML.	PER CENT
Normal				
1	2.19	1.14	1.05	48
2	1.52	0.80	0.72	47
CML				
1	8.65	7.70	0.95	11
2	5.93	5.19	0.74	12

*Serum $B_{12}CO^{60}$ complex acidified with the *Euglena gracilis* basal medium and then redialyzed to determine the UBBC as described in Methods.

TABLE VI. THE EFFECT OF ACIDIFICATION ON THE ABILITY TO BIND $B_{12}CO^{60}$

SERUM	UBBC (mμg/ML.)			
	pH 7.3	pH 5.5	pH 4.5	pH 3.6
Normal				
1	1.32	1.35	1.22	0.55
2	1.02	0.98	0.88	0.50
3	1.00	1.10	0.83	0.28
4	1.59	—	—	0.81
5	1.54	—	—	0.46
CML				
1	9.78	—	—	8.84
2	11.89	—	—	12.90
3	54.32	—	—	53.00

The above pH levels maintained by buffers of similar ionic strength (0.15) as described in Methods

Effect of pH.—Sera containing $B_{12}CO^{60}$ nondialyzable at pH 7.3 was acidified and then dialyzed against the *Euglena gracilis* basal medium pH 3.6. A large fraction (47 to 48 per cent) of the $B_{12}CO^{60}$ nondialyzable at pH 7.3 became dialyzable or unbound on acidification (Table V). On acidification of CML sera a much smaller fraction of the $B_{12}CO^{60}$ was rendered dialyzable. The effect of varying pH levels (3.6 to 9.2) on $B_{12}CO^{60}$ binding was investigated. Binding by normal sera was not affected by pH levels from 5.5 to 9.2. At pH 4.5 a slight decrease in $B_{12}CO^{60}$ binding was found while at pH 3.6 a marked decline occurred (Table VI). By contrast, $B_{12}CO^{60}$ binding by CML sera was unaffected at pH levels of 3.6 (Table VI).

TABLE VII THE BINDING OF $B_{12}CO^{60}$ BY THE SEROMUCOID FRACTION OF NORMAL AND CML SERA

SERUM	SERUM UBBC (mμg/ML)	SEROMUCOID UBBC (mμg/ML)	SEROMUCOID UBBC / SERUM UBBC × 100 (%)
Normals (6)*			
Mean ± S D	2.03 ± 0.14	0.26 ± 0.10	13 ± 5
Range	1.52 - 2.64	0.13 - 0.37	5 - 18
CML			
1	5.81	2.78	48
2	10.19	7.50	74
3	8.29	6.28	76
4 a)†	14.92	9.40	63
b)	28.00	24.50	88
5	4.72	1.46	31
6	4.84	2.17	45
Mean ± S D	10.97 ± 8.33	7.74 ± 7.97	65 ± 20
Range	4.72 - 28.00	1.46 - 24.50	31 - 88

*Refers to number of patients

†Same patient studied during different relapses

TABLE VIII THE EFFECT OF THE PREHEATING OF SERA AND THEIR SEROMUCOID FRACTIONS ON THEIR ABILITY TO BIND $B_{12}CO^{60}$

SERUM	UBBC (mμg/ML) CONTROL	UBBC (mμg/ML) HEATED	UBBC AFTER HEAT / CONTROL UBBC × 100 %
Normal Sera			
1	2.34	1.19	51
2	1.52	0.70	46
3	1.72	0.61	36
4	2.08	0.84	40
5	1.87	0.69	37
CML Sera			
1	5.81	4.89	84
2	8.10	6.59	81
3	10.19	8.60	84
4	23.90	21.50	90
CML Seromucoid			
1 a)	4.65	4.35	94
b)	4.65	3.80	82
2 a)	2.17	2.17	100
b)*	2.17	5.33	246
3 (1)	1.46	1.89	130
4 (1)	23.90	22.90	96

*Samples heated for 10 minutes at 100° C; all other samples heated at 69 to 73° C for 3 minutes

Seromucoid Binding.—The binding of $B_{12}CO^{60}$ by the seromucoid fraction of normal and CML sera is shown in Table VII. A small amount of $B_{12}CO^{60}$ was bound by the seromucoid of normal sera (mean of 0.26 mμg per milliliter equal to 13 per cent of total serum binding). In CML sera this binding was increased about thirty fold (mean 7.74 mμg per milliliter equal to 61 per cent of total serum binding). The binding of $B_{12}CO^{60}$ by the CML seromucoid was not affected by acid pH levels as low as 3.6. Precipitation of the normal or CML seromucoid-vitamin B_{12} complex with 5 per cent phosphotungstic acid resulted in 95 to 100 per cent recovery of the radiovitamin in the washed precipitate.

The Effect of Heat.—The effect of preheating the serum and/or seromucoid on the subsequent ability to bind added $B_{12}CO^{60}$ is shown in Table VIII. Heating of normal sera to 69 to 73° C. for 3 minutes resulted in a 49 to 64 per cent decrease in binding as compared to a 10 to 19 per cent decrease in binding in CML sera. Heating of CML seromucoid to 69 to 73° C. had no effect on $B_{12}CO^{60}$ binding, and heating to 100° C. had no effect or actually increased $B_{12}CO^{60}$ binding in 3 of the 4 sera tested.

DISCUSSION

A large portion (60 to 77 per cent) of the $B_{12}CO^{60}$ bound by normal serum at pH 7.3, i.e., the nondialyzable radiovitamin, was measured as "free" or unbound in the *Euglena gracilis* assay system. In this assay, performed at a pH of 3.6, "free" B_{12} is defined as the vitamin available to *Euglena gracilis* without preliminary heating of the serum-basal medium mixture. However, at such acid pH levels, 47 to 48 per cent of the $B_{12}CO^{60}$ nondialyzable at pH 7.3 became dialyzable or unbound. Thus, the ready availability for *Euglena gracilis* of a large fraction of the bound $B_{12}CO^{60}$ is due to the acid milieu of the assay system. Since the amount of the bound $B_{12}CO^{60}$ measured as "free" by *Euglena gracilis* exceeded the amount of bound $B_{12}CO^{60}$ rendered dialyzable by acidification, direct utilization of bound $B_{12}CO^{60}$ by *Euglena gracilis* without preliminary heating must also occur. Gräsbeck[12] has shown that vitamin B_{12} bound to gastric juice was unavailable for adsorption by *Lactobacillus leichmannii* at pH 7 to 8 but was readily adsorbed below pH 5. Furthermore, it has been shown that other microorganisms can utilize the vitamin B_{12} bound to intrinsic factor preparations without preliminary treatment at pH levels above 6.6.[13, 14] The data cited above emphasize that what is measured as "bound" vitamin in a dialysis or an ultrafiltration system may not be "bound" in a microbiologic assay system. The importance of pH in any type of assay system for the measurement of serum B_{12} binding is also indicated by the present findings. The variable results obtained by other investigators as to the principal binding proteins of normal serum for added B_{12} can probably be ascribed to the different methods of determining vitamin B_{12} binding employed in these studies.[6, 8]

Little, if any, of the vitamin B_{12} of normal serum is measured as "free" by *Euglena gracilis* assay.[6, 15] Thus, the endogenously bound vitamin differs from the vitamin bound in vitro with respect to its availability for *Euglena gracilis.* Differences in the chemical nature of the binding between the in vivo and in vitro situations are suggested by this finding. Furthermore, in vivo the vitamin is primarily associated with the alpha-1 and alpha-2 globulins,[6, 7] whereas in vitro the vitamin is primarily bound by the beta and alpha-2 globulins of normal serum.[8] Doubt as to the physiologic significance of the in vitro binding is suggested by the above findings. Thus, the binding of $B_{12}CO^{60}$ by the beta globulins in the present in vitro system may only represent a "spill over" from the saturated binding sites on the physiologic binding proteins, i.e., the alpha 1 and alpha 2 globulins. Attempts to check this hypothesis using concentrations of $B_{12}CO^{60}$ within the binding capacity of the alpha globulins have been unsatisfactory due to technical limitations. However, small amounts of "free" B_{12}, as measured by *Euglena gracilis* can be found in normal sera[6, 15] and B_{12} activity has been found associated with the beta globulins and albumin fractions of normal serum.[6] Thus, the binding of $B_{12}CO^{60}$ by the beta globulins and albumin fractions observed in vitro also occurs in vivo. The physiologic significance of these findings remains to be elucidated. By contrast to normal sera, the $B_{12}CO^{60}$ bound in vitro by CML sera is largely unavailable to *Euglena gracilis* without preliminary heating, and thus is similar in this respect to the vitamin bound in vivo.[1]

The seromucoid fraction of CML serum was capable of binding large amounts of $B_{12}CO^{60}$ in CML patients. In some sera, the seromucoid accounted for the entire increase in $B_{12}CO^{60}$ binding over normal values. Two factors relating to the method of preparation of the seromucoid could influence the amount of $B_{12}CO^{60}$ bound by the seromucoid. Following perchloric acid precipitation of the serum proteins approximately 30 per cent of the seromucoid is lost by coprecipitation.[11] Secondly, sulfosalicylic acid may liberate the endogenous vitamin B_{12} from the precipitated serum proteins with the liberated vitamin then bound by the seromucoid. Since both factors would decrease the binding capacity for additional $B_{12}CO^{60}$ the $B_{12}CO^{60}$ binding by the seromucoid fraction may be even higher than that measured.

The seromucoid fraction of serum moves with the alpha 1 and alpha 2 globulins on electrophoresis at pH 8.6.[16, 17] The alpha 1 and alpha 2 globulins are also associated with (1) the endogenously bound vitamin B_{12} of normal and CML serum[1, 6] (2) the vitamin B_{12} bound following parenteral administration of the vitamin to normal subjects and patients with CML[18] and (3) the exogenous $B_{12}CO^{60}$ bound in vitro by CML sera.[8] These findings suggest that the seromucoid, or a moiety thereof, may be responsible for the binding of the endogenous and exogenous vitamin B_{12} of CML serum. By the use of anion ion exchange resins, it has been established that the endogenous vitamin B_{12} of CML serum is not bound by the alpha 1 acid glycoprotein,[19] the main protein constituent of the seromucoid.[16] Since the seromucoid contains at least 3 different proteins,[16] the endogenous B_{12} binding protein could still be a constituent of the seromucoid.

The increased $B_{12}CO^{60}$ binding capacity of the CML seromucoid can be due to (1) an increase in the concentration of a B_{12} binding substance present in the normal seromucoid, (2) an increase in the number of B_{12} binding sites of a B_{12} binding protein normally found in the seromucoid, or (3) a B_{12} binding substance foreign to the normal seromucoid. The increase in the concentration of the seromucoid fraction in CML[20, 21] is in favor of the first hypothesis. However, an increase in seromucoid concentration has been found in many diseases in which serum B_{12} binding is not increased.[20, 21] The present data is obviously insufficient to establish which of these hypotheses is correct.

The seromucoid fraction of serum contains a sturdy group of substances as demonstrated by the various methods used in its separation from the serum proteins, i.e., deproteinization of the serum by heating[22, 23] or precipitation of serum proteins with sulfosalicylic acid or perchloric acid.[11] Similarly, the B_{12} binding properties of these substances are not affected by relatively drastic chemical treatment, i.e., heating to 100° C. or acidification. Furthermore, the $B_{12}CO^{60}$-seromucoid complex is not disrupted when precipitated by 5 per cent phosphotungstic acid in 2 N HCl. The minimal effects of heat and of acidification on $B_{12}CO^{60}$ binding by CML whole sera are due to the increased binding by the resistant seromucoid fraction. The unavailability of the bound $B_{12}CO^{60}$ of CML sera for *Euglena gracilis* without heating is probably also explained by the increased seromucoid binding.

It is of interest that two substances with high vitamin B_{12} binding properties are mucoproteins, i.e., the intrinsic factor of Castle[24, 25] and the sow milk whey protein.[26] Evidence both for[27-30] and against[31-33] the presence of an extragastric intrinsic factor-like substance present in normal serum but absent in pernicious anemia serum has been presented. The available data do not suggest any relationship of the $B_{12}CO^{60}$-binding substances of the CML seromucoid to intrinsic factor. Thus, heating to 100° C. did not affect its $B_{12}CO^{60}$ binding ability in contrast to its effect in gastric juice.[34] An actual increase in binding by some seromucoid fractions was noted after heating, an effect not found with any purified or crude intrinsic factor preparation.[35] The substances of CML serum with increased $B_{12}CO^{60}$ binding properties move as alpha-1 and alpha-2 globulins on electrophoresis at pH 8.6,[8] whereas the intrinsic factor activity in human gastric juice was found slightly ahead of the beta globulins under similar conditions.[35]

SUMMARY AND CONCLUSIONS

1. Binding of added cobalt^{60}-labeled vitamin B_{12} ($B_{12}CO^{60}$) by normal and CML sera was studied by dialysis techniques. Added $B_{12}CO^{60}$ was rapidly bound and once bound did not exchange with additional "carrier" vitamin B_{12} in both normal and CML sera.

2. Of the bound $B_{12}CO^{60}$ of normal sera, i.e., the $B_{12}CO^{60}$ nondialyzable at pH 7.3, 60 to 77 per cent was measured as "free" by *Euglena gracilis* assay, whereas only 9 to 13 per cent was so measured in CML sera. At the acid pH at which the *Euglena gracilis* assay is performed (pH 3.6), 47 to 48 per cent of the bound $B_{12}CO^{60}$ of normal sera became dialyzable as compared to 11 to 12 per cent in CML sera.

3. $B_{12}CO^{60}$ binding by normal sera was unaffected by pH levels above 4.5 but a marked decline in binding was found at pH 3.6. In contrast, at pH 3.6, $B_{12}CO^{60}$ binding by CML sera was unaffected.

4. Preheating of normal sera to 69 to 73° C. for 3 minutes decreased $B_{12}CO^{60}$ binding by 49 to 64 per cent as compared to 10 to 19 per cent in CML sera.

5 $B_{12}CO^{60}$ binding by the seromucoid fraction of normal serum was equal to an average of 0 26 mμg per milliliter, constituting 13 per cent of the total serum binding, while binding by the CML seromucoid was equal to 7 74 mμg per milliliter, constituting 61 per cent of the total serum binding Binding by the CML seromucoid was not affected by acidification or heating to 100° C

6 Differences noted between normal and CML sera with respect to (1) the effects of acidification and heating on $B_{12}CO^{60}$ binding and to the (2) availability of bound $B_{12}CO^{60}$ for *Euglena gracilis* are due to the increased binding by the CML seromucoid The increased in vitro binding ability of CML serum for added $B_{12}CO^{60}$ is due to the increased $B_{12}CO^{60}$ binding by the seromucoid fraction of serum, the increased in vivo binding capacity of CML sera for vitamin B_{12} may be attributed to a similar mechanism

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LABORATORY METHODS

A CAPILLARY TEST TUBE TECHNIQUE FOR THE DETERMINATION OF CAPILLARY WHOLE BLOOD PROTHROMBIN TIME

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THE repeated determination of the prothrombin time in infants, especially in the first few months of life, presents serious problems of technique. The repeated sampling of the usual amounts of blood required for the Link-Shapiro modification of the Quick test for prothrombin, as is generally used for this determination in our laboratory, requires repeated venepunctures with the withdrawal of amounts of blood that are proportionally huge in relation to the small reserve available to the newborn infant. Since such tests are most often required of the abnormal infant in contrast to a healthy baby able to withstand such vigorous testing, much investigation has gone into this phase of minimizing the trauma of the test to the infant. Several different types of micromethod determination of the prothrombin time especially for use in infants have been described in an effort to solve the dilemma presented.[1-9] However, these tests prove quite complex and cumbersome in their requirement for the exacting care and stringent conditions in handling and testing the small quantities of blood or plasma sample and thus none offer a convenient and easily available bedside method for the accurate and immediate determination of the prothrombin time of the blood. This laboratory investigated the accuracy and utility of a bedside capillary test tube technique suggested by one of us (E. G.) for such a purpose.

METHODS

A clean glass capillary tube, measuring 1 mm. internal diameter and 3 cm. long, is marked off in 1 cm. intervals. A room temperature mixture of 0.0125 M calcium chloride and thromboplastin solution* is drawn up to the 2 cm. marking and then very freely flowing blood, as obtained from a deep skin puncture in a digital tuft, ear lobe, or heel, is drawn to the 3 cm. mark. The tube is then immediately grasped between the thumb and forefinger to close off the system and inverted repeatedly until the end point of a definite precipitate is reached. This precipitate is easily identified with practice. The exact

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*Solu-Plastin, Schieffeling & Company, 16-30 Cooper Square, New York 3, N. Y.

Table I. A Comparison of Prothrombin Time Determinations Between the Modified Quick Test Using Plasma and the Capillary Test Tube Using Oxalated Venous and Capillary Blood in Infants

PATIENT NUMBER	PROTHROMBIN TIME IN SECONDS		
		CAPILLARY TEST TUBE METHOD	
	PLASMA METHOD	VENOUS OXALATED BLOOD	CAPILLARY BLOOD
1	22	18	16
2	20	18	17
3	17	17	17
4	26	22	23
5	15	16	15
6	15	20	19
7	15	18	17
8	16	18	18
9	16	17	17
10	17	19	18
11	16	19	17
12	16	16	18
13	22	20	19
14	19	20	19
15	18	21	19
Mean ± S.D. of Mean	18.6 ± 1.9	18.0 ± 3.6	18.0 ± 1.7

Table II. Comparison of Prothrombin Time Determinations Between the Modified Quick Test Using Plasma and the Capillary Test Tube Using the Same Oxalated Venous Blood Sample

PER CENT DILUTION	PLASMA METHOD (REAGENTS AND PLASMA AT 37° C.)	CAPILLARY TEST TUBE METHOD: REAGENTS AND WHOLE BLOOD AT		
		22° C.	30° C.	37° C.
100	15*	15*	19*	15*
75	19	19	21	16
57	21	21	22	18
42	23	21	23	22
32	28	22	24	24
24	32	24	29	26
18	38	25	30	32
13	45	34	35	37
10	50	42	52	45

*Values given in seconds.

Table III. A Comparison of Capillary Tube Prothrombin Time Determinations Between Oxalated Venous Blood and Capillary Blood in Adults

PATIENT NUMBER	PROTHROMBIN TIME IN SECONDS IN DUPLICATE	
	OXALATED VENOUS BLOOD	CAPILLARY BLOOD
1	20.0-19.0	19.5-19.5
2	14.5-14.0	14.0-14.0
3	17.5-16.0	18.5-18.0
4	15.0-16.0	16.0-15.0
5	18.0-18.5	17.0-16.0
6	15.0-16.0	15.5-14.5
7	17.0-16.0	17.0-17.5
8	17.0-17.0	19.0-19.0
9	16.0-16.0	17.0-18.0
10	17.0-17.0	18.0-18.5

timing of the test is from the time of the initial mixing of the blood with inverting the tube to this end point of a definite and easily visible precipitate. Table I tabulates the results of the comparisons of the Link-Shapiro modification of the Quick test for prothrombin time, using a plasma substrate, is compared to the capillary test tube technique using both oxalated venous and capillary blood samples on the same patients. The mean value for the prothrombin times *of each technique is approximately 18 seconds*. The variations within each test group, as determined by the standard deviation from the mean are equally and surprisingly similar, considering the usual range of error anticipated in biologic tests.

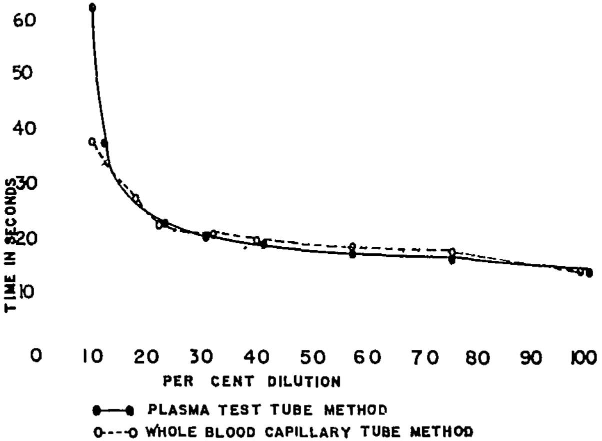

Fig. 1.—A comparison of prothrombin time dilution curves between the Link-Shapiro modification of the Quick test using plasma and the capillary tube method using oxalated venous blood.

RESULTS

Since this testing is done at room temperature rather than in the usual environmental temperature of 37° C., the variation of the capillary test tube technique at several different temperatures was investigated and is reported in Table II. The same sample of oxalated venous blood was used for each determination. First, a dilution curve using the modified Quick test on a plasma substrate at 37° C. was determined. Next, the capillary test tube method was used with the oxalated whole blood as a substrate maintaining the temperature of the blood and substrates at 37° C., 30° C., and finally at room temperature of 22° C. Reference to Table III confirms the accuracy of the capillary test tube technique with good correlation at all temperature ranges for the concentrated specimens to the 24 per cent dilution. From this point to the 10 per cent dilution, there is a larger margin of variation than would be permissible for the 22° C., but less so and in an acceptable range for the 30° and 37° C. runs. Fig. 1 describes a demonstration using a different oxalated blood specimen. This figure compares the modified Quick test on the plasma substrates run at 37° C., while the capillary test tube technique was run at 22° C. The curves are very similar, with the tendency to lose accuracy only at the highest dilutions.

DISCUSSION

The major source of error in this capillary test tube technique lies in the need for a *free flow* of capillary blood. Any modicum of squeezing or local trauma that may be used to speed the flow of blood from the puncture site, no matter how trivial it may seem, creates marked variations of the prothrombin time in both directions. If the flow of blood from the puncture wound is too slow to be of use, a new site should be punctured for the determination rather than to attempt any manipulation whatsoever, no matter how gentle it would seem. This test, therefore, demands special familiarization with this aspect. The skill in recognition of the precipitant end point is quickly obtained in use and does not present a problem.

The convenience and accuracy of this capillary test tube technique for the determination of the prothrombin time using capillary blood has offered us a very useful tool to investigate prothrombin times in any special situation where adequate quantities of freely flowing venous blood cannot be obtained. This test is *not* recommended to supplant the usual techniques of prothrombin time determinations when they are convenient to do. We have had great success in using this test in the newborn nurseries for a specific research purpose and have used it in adults as well with equally satisfactory results (Table III). The bedside accuracy of this method plus the easy portability of the minimum equipment needed makes it potentially of great service for the speedy determination of prothrombin times in the hospital clinic or for home bedside use. The increasing use of long-term antiprothrombin drugs for anticoagulation therapy may require the use of such an inexpensive relatively accurate technique that can be used without requiring centrifugation or incubation of the samples, yet give an exact record of the blood prothrombin content.[6] The apparent simplicity of the test, however, should not minimize the very close attention to the important details discussed above.

SUMMARY

A bedside micromethod for the testing of prothrombin times using capillary blood as a substrate in a capillary glass test tube system at room temperature is described. This technique has been compared statistically with the usual Link-Shapiro modification of the Quick test in newborn infants as well as adults and found as accurate.

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A SEMIMICROTECHNIQUE FOR IRON^{59} BETA DETERMINATIONS IN BIOLOGIC MATERIALS

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IRON^{59} has been employed at the Radiobiological Laboratory of the University of Texas and the United States Air Force to evaluate iron incorporation into maturing erythrocytes of the monkey when the iron was administered in various forms,[1] and as an index of erythropoietic damage in monkeys exposed to mixed ionizing radiation.[2] In these studies, a modification of the method of Hennessy and Huff,[3] which utilizes crystal scintillator counting of the Fe^{59} gamma emissions, has been employed.

In hematologically abnormal or irradiated animals, erythropoietic activity may be markedly reduced or absent. To effect good counting under such conditions, it has sometimes been necessary to use 3.0 ml. of blood. In small animals, both sample volume and sampling frequency may be limiting.

The mode of decay of Fe^{59} to Co^{59} is by paired beta-gamma emission with mean energies of 1.1 mev. (gamma) and 0.46 mev. (beta), or 1.3 mev. (gamma) and 0.26 mev. (beta).[4] Consequently, the 1:1 beta-gamma ratio permits the use of either type of emanation in detection. Thin window or gas flow counting has been used to detect iron^{59} beta radiation,[5, 6] although recent work has usually employed gamma counting techniques in which crystal scintillators are used.[7, 8] Both techniques have inherent limitations; sample absorption can be limiting with the low-energy betas, while crystal scintillator absorption of relatively high-energy gammas frequently results in reduced counting efficiency.

This paper describes a technique for digesting iron-containing components of biologic systems, reducing and complexing the iron, and selectively extracting and concentrating the complex. In this form, total iron may be measured spectrophotometrically and then introduced into a liquid scintillator solution which permits beta counting of any Fe^{59} present.

MATERIALS AND METHODS

The care, maintenance, and clinical history of animals at this laboratory have been described.[9-12] Methods of preparing specific radioiron compounds, injection and counting techniques, and typical iron incorporation curves of these monkeys have also been reported in detail.[1, 2]

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To retain comparability with prior studies, two male *Macaca mulatta* monkeys analogous in age, weight, and clinical history to animals used in cited references were employed *in this study.*

A 1.0 ml. blood sample previously reserved for hematologic determinations was used for determining radioiron incorporation by the beta counting method under test, while the remainder was counted by the crystal scintillation method. This procedure tested replication of gamma counting data against the extensive previous work, and determined the ability of the beta technique to measure the identical phenomenon.

Beta Counting of Fe^{59} in a Liquid Scintillator System.—The procedure for digesting iron from protein complexes in blood, and selectively extracting it in recomplexed form is a modification of the basic technique of Peterson.[14]

Either whole blood or subfractions may be used. The technique permits spectrophotometric determination of total iron. In addition, the same solution may be introduced into liquid scintillator solutions through secondary and tertiary solvent systems. In the tertiary solvent system, any iron^{59} present may be detected by counting photons resulting from the beta emanations.

Preparation of Whole Blood for Beta Counting.—

A. Reagents

1. Concentrated sulfuric acid, reagent grade.
2. Trichloroacetic acid (TCA), reagent grade, 25 per cent solution in deionized water.
3. Sodium acetate, reagent grade, saturated solution in deionized water. Maintain excess sodium acetate in bottom of flask.
4. Thioglycollic acid, reagent grade.
5. 1,10-Phenanthroline (orthophenanthroline),* 0.5 per cent solution in isoamyl alcohol (IAA).†
6. Toluene.‡
7. Absolute ethanol (petroleum derived for low C^{14} content).
8. Stock scintillation solution: 2,5-diphenyloxazole (5.45 Gm.) and 2,2′-p-phenylene-bis(5-phenyloxazole) (0.18 Gm.) *in 1 liter of toluene.*

B. Procedure

1. Place 1.0 ml. concentrated sulfuric acid and a glass bead into a 50 ml. Pyrex centrifuge tube, and add 0.2 ml. whole blood. Under an exhaust hood, heat the mixture over a microburner until heavy white fumes appear. After 5 minutes, turn off the burner, and cautiously add 5.0 ml. deionized water while gently shaking the reaction tube. Allow the reaction mixture to cool to room temperature.

2. Add 0.1 ml. thioglycollic acid to the reaction mixture.

3. Add 2.0 ml. 25 per cent TCA to the reaction mixture and allow the precipitate to settle.

4. Add 10.0 ml. saturated sodium acetate (final pH should approximate 5.0) and centrifuge for 10 minutes at about 825 g.

5. Decant the clear supernatant into a clean 25 ml. glass-stoppered graduate cylinder. Add about 5 ml. IAA, *stopper the cylinder, shake vigorously, and discard the IAA layer.* This step removes IAA-soluble quenching agents from the system.

6. Add 5.0 ml. of the 0.5 per cent orthophenanthroline-IAA solution. Stopper and shake as in Step 5. This IAA layer, containing the red-orange iron-orthophenanthroline complex, may be transferred by capillary pipette.

7. If measurement of total iron is desired, this solution may be read spectrophotometrically at 512 mμ and compared to a standard curve. If no spectrophotometric *determination is required, 2.0 ml. of the solution may be transferred directly to a Wheaton* 5-dram Crystal Lite counting vial with polyethylene cap.

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*G. Frederick Smith Chemical Company.

†Baker Chemical Company.

‡Phillips Petroleum Company.

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Absorption spectra of all components of the liquid counting system were determined on the Beckman DU spectrometer, separately and in all possible combinations. These spectra were then compared to the emission spectra of the 2,5 diphenyloxazole and 2,2′ p phenylene bis(5 phenyloxazole) as given by Hayes and associates[16] Absorption and emission spectra evidenced a "tail" overlap in the region of 500 mμ which confirmed the observations made on model systems.

Initial beta spectrum (photon yield) determinations accomplished on the Tri Carb liquid scintillation spectrometer evidenced a marked "shift" phenomenon characterized by erratic count rates from the same sample. However, when the sample was flushed with argon[13] and adequately sealed the relative photon yield for the sample was stabilized. This apparent oxygen effect—now known to be attributable to oxidizable impurities sometimes found in two of the reagents—requires that samples be counted within about 6 hours after flushing and sealing.

Under the stipulated counter conditions coincidence counting efficiency of blood derived Fe^{59} consistently ranges from 35 to 40 per cent when intercompared with NBS $FeCl_3$ secondary standards. Because the iron is homogeneously distributed in the counting system, and because of its intercomparability with the NBS standard, the system may be regarded as an "absolute" beta counting technique.

Iron⁵⁹ Incorporation.—Incorporation curves determined by both the beta and gamma counting techniques are shown in Fig. 1. The gamma counting data (obtained using the Nancy Wood crystal scintillation counter, Model SC 2L, and the calculations of Hennessy and Huft) reflect individual incorporation curves which are closely comparable in both rate and amplitude to individual animals of earlier studies[1, 7] For illustrative purposes the average value has been plotted.

TABLE I NET COUNTS PER MINUTE PER MILLILITER OF BLOOD AS DETERMINED BY BOTH BETA AND GAMMA COUNTING TECHNIQUES

DAY	NANCY WOOD CRYSTAL SCINTILLATOR* (MODEL SC 2L) ANIMAL NUMBER 625	NANCY WOOD CRYSTAL SCINTILLATOR* (MODEL SC 2L) ANIMAL NUMBER 872	TRI CARB† (PACKARD LIQUID SCINTILLATION SPECTROMETER) ANIMAL NUMBER 625	TRI CARB† (PACKARD LIQUID SCINTILLATION SPECTROMETER) ANIMAL NUMBER 872
0 Injection assay	6,528	6,315	19,873	20,000
1	741	344	1,213	1,663
2	991	1,637	4,775	5,063
4	2,394	3,778	10,013	11,150
7	3,744	4,825	12,375	12,363
9	3,680	4,530	12,088	14,138
11	3,678	4,730	11,450	13,788
14	3,645	4,937	11,289	14,340

*Accomplished on blood volume of 1.0 ml or greater.
†Accomplished on the equivalent of 0.08 ml of blood.

Table I shows the net counts per minute per milliliter whole blood for each animal as determined by the beta and gamma counting techniques. These data were accrued prior to learning that quenching agents from the blood (lipids?) were being carried into the counting system. Extraction of these materials prior to step 6 allowed blood-derived systems to be equated with model systems. Since the incorporation data in Fig. 1 were calculated from the proffered data, Table I very probably underrates the power of the beta counting method. Only the counting efficiency appears to be affected by the presence of the quenching materials.

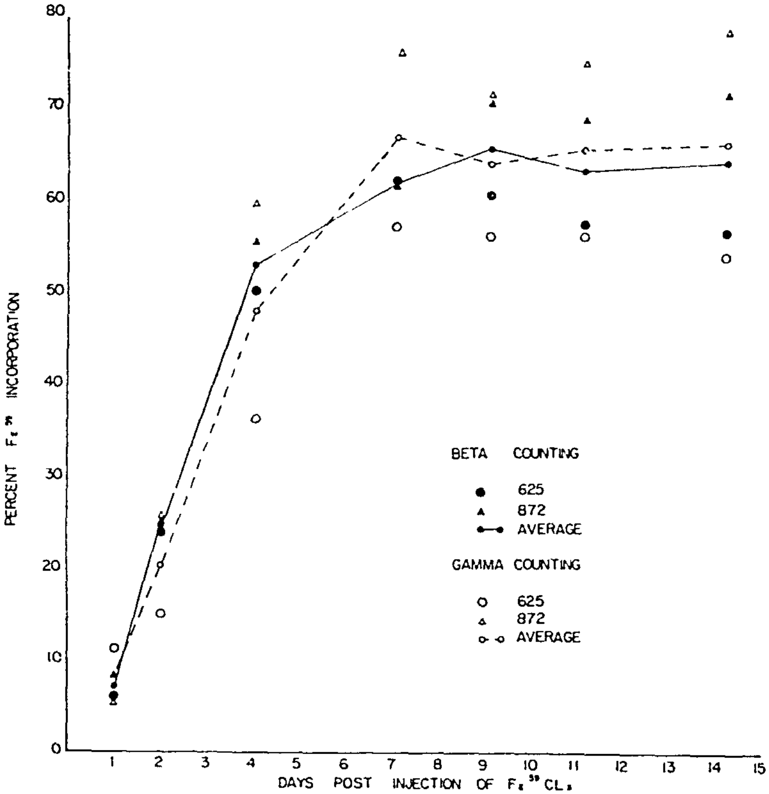

Fig. 1.—Comparison of iron^{59} incorporation curves as determined by both beta and gamma counting methods.

DISCUSSION

The liquid scintillation method of counting Fe^{59} beta emissions permits reduction in blood sample volume when radioiron incorporation is used as an index of erythropoietic activity. Reductions in initial sample size range from $\frac{1}{5}$ (1.0 to 0.2 ml.) up to $\frac{1}{15}$ (from about 3.0 to 0.2 ml. whole blood).

The technique is capable of reproducing the incorporation patterns obtained by the commonly used gamma counting technique. Although only 2 test animals were used in this evaluation, the resultant data are in excellent agreement with both the concurrent gamma data and the extensive previous work. Additionally, it is apparent that there is less divergence between beta counts from the individual animals than is exhibited by the individual gamma counts. Consequently, a greater resolution is suggested which is probably attributable to both the increased counting efficiency of the technique and the greater precision inherent in coincidence counting.

Efficiency of the beta counting technique is from 35 to 40 per cent in blood derived systems Low level crystal scintillator counting of the associated 1 1 and 1 3 mev gammas approximates an efficiency of 7 per cent in this laboratory Net increase in efficiency is, therefore, from five- to sixfold The beta counting technique for Fe^{59} was developed specifically for use with small laboratory animals in which blood sample volume and frequency may become limiting and was tested on apparently normal animals If extremely low incorporation of the iron causes counting difficulties, the total iron in the counting system may be increased by adding more than 2 0 ml of the iron orthophenanthroline complex (in the IAA extract) to the counting system with a concomitant reduction in the amount of ethanol used However, the efficiency of counting must decline as concentration of the complex in the counting solution is increased, fall off in efficiency approximates linearity over the concentration range thus far investigated

The technique suggests potential clinical application in cases where infants and small children are under study for blood dyscrasias etc When coupled with spectrophotometric determinations of total iron in blood its subfractions, or in other biologic components the beta counting technique lends itself to rapid calculation of specific activity which is required in the study of ferrodynamics

SUMMARY

After acid digestion of iron containing components of biologic systems, the iron may be selectively complexed and extracted from the digest The colored complex is of adequate stability to permit spectrophotometric measurement of total iron If iron^{59} is also present in the system, the same colored complex may be introduced into a liquid scintillator solution and the beta emanations counted with an efficiency of from 35 to 40 per cent at the concentrations of iron derived from blood The technique offers a net increase in counting efficiency of from five to sixfold as compared to the commonly used gamma counting technique, a reduction in size of the initial sample up to one fifteenth, and a substantial, but unquantitated, increase in the precision of counting

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THE DETERMINATION OF FREE AND TOTAL CHOLINE IN SERUM AND ITS APPLICATION TO THE PARTITION OF SERUM PHOSPHOLIPIDS

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THIS paper deals with three problems (1) the validity of the microbiologic assay of total choline in serum, (2) the measurement of free choline in serum, and (3) the partition of serum phospholipids into those containing choline and those that do not

Bligh[1] and Appleton and associates[2] believe the microbiologic assay originally described by Horowitz and Beadle[3] and used by Luecke and Pearson[4] for the measurement of the choline content of plasma is not specific for choline However, Duell believes this method is quite sensitive and specific Review of the original articles by Horowitz and Beadle[3] and Luecke and Pearson[4] indicate several studies were omitted that are necessary to establish the validity of this type of assay The results of experiments designed to test the validity of the microbiologic assay are reported here and indicate the measurement of *total* choline in serum is specific, accurate, and reproducible

The measurement of *free* choline in serum was also undertaken Bligh[1] has summarized beautifully the problems inherent in the measurement of free choline in serum Using a biologic method he found plasma contained 1 to 2 μg choline per milliliter Appleton and co-workers[2] measuring a periodide precipitate of choline in ultraviolet light found an average of 4 4 μg choline per milliliter of plasma These are in contrast to the findings of Luecke and Pearson[4] of 44 to 75 μg choline per milliliter of plasma using the microbiologic method The results presented below indicate that contrary to the findings of Luecke and Pearson[4] there is no detectable *free* choline in serum using the microbiologic method

There is also disagreement about the quantity of choline containing and noncholine containing phospholipids in human plasma Earlier work, summarized by Taurog and associates[6] indicated 20 to 40 per cent of serum phospholipids are noncholine containing Albrink[9] more recently has confirmed this earlier work However, Taurog and associates[6] found that only 0 to 8 per cent of serum phospholipids do not contain choline These findings were confirmed by Hack[7] and Sinclair,[8] although review of their data indicates that in some of the plasma samples examined as much as 8 to 17 per cent of the serum phospholipids are free of choline Our studies to date in 38 normal

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